Review Letter

APPROACHES TO THE STUDY OF ENZYME MECHANISMS LACTATE DEHYDROGENASE

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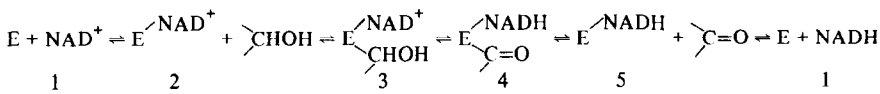
1. Introduction

As the detailed description of chemical, structural and kinetic features of certain enzymes and their reactions becomes more and more sophisticated a review article should concern itself with the answers to quite specific questions. In the present discussion the aspect of enzyme reactions considered is the definition of the different enzyme intermediates and of the individual steps of enzyme–substrate combination and catalysis. Enzyme–substrate combination and the associated phenomenon of the formation of the "reactive" enzyme–substrate complex are here regarded as the specific and biological process. The subsequent catalytic process of the interconversion of the reactive complexes with substrates and products is more the concern of those who are interested in the physical-organic chemistry of reaction mechanisms and may be regarded as an open ended question. However, part of the exercise of defining the different enzyme–substrate complexes is to characterise the groups of the enzyme which play a direct role in the interconversion of the enzyme complexes.

We are presenting the results of experiments with pig heart lactate dehydrogenase and liver alcohol dehydrogenase to illustrate the application of chemical, spectroscopic and fluorimetic methods which can now be combined with transient kinetics to characterise the different enzyme intermediates. The results will be interpreted in terms of a plausible mechanism which is at best incomplete but may even need revision as the resolution of the methods is improved.

Early studies of the stereochemistry, steady state kinetics and equilibria of lactate and alcohol dehydrogenases resulted in a general acceptance of a reaction mechanism involving ternary complexes in which the pyridine nucleotides bind first and the smaller substrates afterwards [1, 2]. The work 'nucleotide' will be used for NAD^+ and NADH. The word 'substrate' will be used for alcohol, lactate, acetaldehyde and pyruvate (scheme 1).

Lactate dehydrogenase does not form a complex with pyruvate in the absence of NAD(H) nor a complex with lactate with a dissociation constant of less than 1 mM [3]. Similarly, no complexes are formed with the substrate analogues oxamate and oxalate in the absence of nucleotides [4]. Kinetic observations also showed [5] that the rate of dissociation of NADH from the ternary complex with oxamate was at least one hundred times slower than that observed with the binary ENADH complex. Complexes of alcohol dehydrogenase with alcohol and acetaldehyde are



Scheme 1. The ordered binding of nucleotides and substrates to alcohol and lactate dehydrogenases. The point at which the proton is taken up is not shown. >CHOH represents alcohol and lactate, >C=O represents acetaldehyde and pyruvate. Abortive complexes (both nucleotide and substrate at the same oxidation state) are known but omitted from the scheme.

not required to explain the steady-state kinetic data of Dalziel [6]. Binary enzyme–substrate complexes are, therefore, excluded from our discussions.

2. Equilibrium measurement of partial reactions

2.1. *Binary nucleotide complexes*

NADH has an absorption maximum at 340 nm due to the dihydronicotinamide ring which is shifted to 325–330 nm on binding to many dehydrogenases [7, 8]. The observation of difference spectra shows that each subunit binds one NADH.

$$E + NADH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^{NADH}$$

$$K_{E,NADH} = \frac{[E]\cdot[NADH]}{[E^{NADH}]} = < 1\ \mu M$$

Equilibrium constants for the above reaction are under 1 μM at neutral pH, and it is necessary to be able to measure the formation of the E^{NADH} complex with total enzyme binding sites concentration (E) of 0.2 μM if there is to be sufficient unbound NADH in the solution to examine the equilibrium accurately. Fluorescence techniques are sufficiently sensitive.

Pure NAD^+ is not appreciably fluorescent. When excited at 340 nm the dihydronicotinamide ring of NADH fluoresces at about 460 nm [9]. The fluorescence of bound NADH is increased to the same extent at each of the 4 sites of the lactate dehydrogenase molecule. Impurities in commercial NADH compete with the NADH binding sites at high enzyme concentration and one cannot overemphasize the necessity of using NADH which has been freshly rechromatographed on ion-exchange cellulose [10, 11]. These technical difficulties can be avoided if the titration with NADH is performed in the presence of a substrate analogue (isobutyramide for alcohol and oxamate for lactate dehydrogenase [12, 13]). Direct proof that the quantum yield of bound NADH is the same for each of the two sites of the dimer or for each of the four sites of the tetramer is then easily obtained since only the binding of NADH and not of the impurities is tightened by the presence of the substrate analogue. The changes in NADH fluorescence can be described accurately by a single macroscopic dissociation constant independent of protein concentration [10, 14–16] and thus all the NADH binding sites on each dehydrogenase molecule are independent, indistinguishable and energetically equivalent. The binding of NADH to the two dehydrogenases depends upon pH in much the same manner [10, 16, 17]. The dissociation constants are largely independent of pH from pH 5.5 to pH 8. Above this pH the binding of NADH becomes rapidly weaker up to about pH 11 and in more alkaline solutions the enzymes denature.

The formation of a complex between dehydrogenases and NAD^+ is less easy to study by perturbation of physical properties. Pure NAD^+ neither appreciably quenches the tryptophan fluorescence of lactate dehydrogenase, nor fluoresces itself, nor gives an easily measurable difference spectrum on binding [10] (except when NADH is formed from contaminants). The E^{NAD^+} complex of lactate dehydrogenase was detected by Takenaka and Schwert [3] using an ultracentrifuge technique and because of the high concentrations of enzyme used this result is particularly reliable. Measurements of NAD^+ binding at low enzyme concentrations are liable to many errors. NADH binds 300-fold tighter at some pH values than NAD^+ so that any NADH or NADH-like adducts present in the system as impurities will cause large errors. NADH is easily formed from the lactate introduced into the system as sweat. Commercial NAD^+ is often contaminated by dihydronicotinamide like NAD-adducts with sulphite or acetone. Only when extreme precautions are taken is it possible to measure NAD^+ binding by displacement of NADH from dehydrogenases [10, 14]. Neither the ultracentrifuge technique nor the gel-filtration method of measuring NAD^+ binding are suitable for studies as extensive as those performed on NADH binding. The affinity of lactate dehydrogenase for NAD^+ is independent of pH between pH 6 and pH 8.5 [10]. Binding of NAD^+ to alcohol dehydrogenase is on the other hand very dependent of pH [14].

Proteins which contain tryptophan fluoresce strongly at about 350 nm when excited at 270–305 nm. The protein fluorescence is quenched when acceptor molecules with an absorption spectrum which overlaps the protein emission spectrum are bound. Radiationless transfer [18] of energy from excited

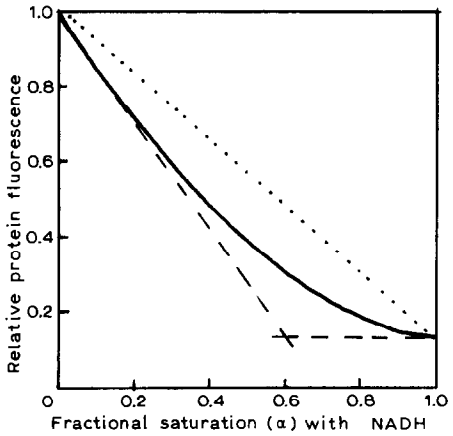


Fig. 1. The non-linear relation of protein fluorescence (F) to the degree of saturation (α) of the NADH binding sites. The curve is described by $F^{1/n} = 1-\alpha(1-x)$ when n = 4 and x = 0.59. The relative fluorescence of LDH tetramers with j molecules of bound NADH is x^j, that is for j = 0, 1, 2, 3 and 4, x^j = 1, 0.59, 0.35, 0.21 and 0.12. NAD^+ does not appreciably affect LDH fluorescence. Excitation was at 305 nm, emission was at about 345 nm (Kodak 98). The initial tangent (dashes) intersects the end point at α = 0.61, as predicted from $(1-x^n)/n(1-x)$ [30].

tryptophan residues in a protein to the bound dihydronicotinamide ring of NADH [19–23] can occur over quite large distances. It has been calculated that the critical distance at which deactivation of excited tryptophan residues by radiationless transfer to NADH equals deactivation by emission is about 2.5 nm [24]. Such long distance interactions mean that excitation energy will be transferred across subunit interfaces and it has recently been shown that the way in which protein fluorescence (F) decreases with increasing degree of saturation (α) of the NADH binding sites contains information about the number (n) of non-interacting binding sites in homopolymeric dehydrogenases [23]. The protein fluorescence is related to the degree of occupancy of the identical NADH binding sites by the equation $F^{1/n} = 1-\alpha(1-x)$. The constant x has been called 'geometric quenching factor' because the relative quantum yields of protein molecules with j molecules of bound NADH are x^j [22]. This is shown in fig. 1. The non-linear, geometric relation between the quantum yields arises because the enzymes examined consist of an approximately symmetrical arrangement of subunits and thus the same fraction (x) of the initial excitation energy will always be transferred to the bound NADH when the (j+1)th molecule of NADH binds. In oligomers of identical subunits there can be a linear relationship between F and α but only when the binding is completely cooperative (positive or negative) or when ligand binding rapidly induces dissociation into monomers [23]. The non-linearity in protein fluorescence has been suspected for some time [20], although it should be emphasised that the non-linearity is not due to non-identity of subunits, or to any negative cooperativity but is simply the result of radiationless transfer of electronic excitation energy from tryptophan residues in one subunit to NADH bound at another subunit in the same molecule. Neglect of the non-linearity can lead to errors in interpretation of both equilibrium and kinetic experiments.

2.2. *Active and abortive ternary complexes*

The chemical evidence for the presence of active ternary complexes of the type shown in scheme 1 has been reviewed [25] and the section on the transient kinetics of dehydrogenases will provide direct evidence. The ternary complex lactate dehydrogenase–NADH–nitrophenylpyruvate has been directly observed [31] and is sufficiently stable for its properties to be examined in some detail.

Abortive ternary complexes are those in which the enzyme forms a complex with a coenzyme and substrate both of which are at the same oxidation state. Lactate dehydrogenase forms both possible abortive ternary complexes. It has long been known that high concentrations of pyruvate inhibit the oxidation of NADH catalysed by lactate dehydrogenase [27–29]. A study of the rate of appearance of the inhibited enzyme and the rate of formation of $E^{NAD^+}_{pyruvate}$ established that the two processes were concomitant [30]. The oxidised abortive ternary complex shows an absorption maximum at 323 nm, quenches protein fluorescence but does not fluoresce at 460 nm.

The reduced abortive ternary complex, $E^{NADH}_{lactate}$, is far less well studied even though it has been recognised for some time [31]. It is twice as fluorescent at 460 nm than the ENADH complex and is more stable below pH 7 (in this respect it resembles other complexes of the type $E^{NADH}_{carboxylate}$). There are reports that excess lactate inhibits lactate dehydrogenase at low pH, although the role of the reduced abortive ternary complex in the steady state of the lactate

dehydrogenase forward reaction at pH 5.5 to 7.5 is not yet clear. Some samples of lactate contain 0.05% pyruvate, and most samples of NAD^+ contain tight binding dihydronicotinamide derivatives. This situation can result in artifacts, especially when it is realised that the overall equilibrium constant is 4×10^{-12} M [32].

2.3. *Ternary complexes with inhibitors*

When NAD^+ is bound to a number of dehydrogenases, the 4-position of the nicotinamide ring is susceptible to nucleophilic attack and ternary complexes of E^{NAD^+} with sulphite [33], cyanide, acetone, pyruvate and many other nucleophiles can be stabilised (for reviews see [1, 25]). These complexes involve the formation of a stable covalent intermediate between the nucleophile and bound NAD^+ and while they have been useful to titrate the concentration of dehydrogenase active sites, they form slowly and should not be confused with complexes of enzyme, coenzyme and substrate analogues which are in rapid equilibrium and can provide analogue information about the enzyme mechanism.

Alcohol dehydrogenase is in rapid equilibrium with its complex with NADH and isobutyramide [12]. This complex is much more fluorescent than E^{NADH} and has been used to titrate the normality of coenzyme binding sites. The dissociation constant of isobutyramide from the $E^{NADH}_{isobutyramide}$ complex is not markedly dependent upon pH. Pyrazole is a competitive inhibitor of alcohol and forms only an unprotonated complex $E^{NAD^+}_{pyrazole}$ which can be detected from its absorption at 300 nm [34].

$$EB: \quad {}^{NADH} + H^+ \overset{pK = 6.8}{\rightleftharpoons} {}^{NADH}EBH^+ \quad + \text{oxamate} \overset{K = 3\,\mu M}{\rightleftharpoons} {}^{NADH}EBH^+_{\text{oxamate}}$$

Scheme 2. Formation of a stable ternary complex between lactate dehydrogenase, NADH and a substrate analogue (oxamate, oxalate, nitrophenylpyruvate and possibly lactate) involves the obligatory protonation of histidine-195. B: is the unprotonated imidazole portion of this amino acid. The pK is the same for all carboxylates but the limiting dissociation constant at low pH varies [26] with carboxylate and isoenzyme and species from which the enzyme was obtained. $K_d = 3\ \mu M$ is for pig heart lactate dehydrogenase.

Lactate dehydrogenase forms many complexes with substrate analogues, of which the complexes $E^{NADH}_{oxamate}$ and $E^{NAD^+}_{oxalate}$ have been most useful in elucidating the mechanism of catalysis [3, 4, 26, 35, 36]. Oxalate is a competitive inhibitor of lactate when measured at moderate concentrations. Oxamate, an isoelectronic analogue of pyruvate, forms a tight complex with E^{NADH}. In this complex the protein fluorescence is quenched by the presence of NADH [26], the normally enhanced fluorescence of bound NADH is quenched to less than that of free NADH at 460 nm, and there is a difference spectrum at 320 nm. Using lactate dehydrogenase Schwert and his group recognised that formation of stable complexes of the type $E^{NADH}_{carboxylate}$ is favoured by protonation of a group of about pK 7 [35] although they also suggested that another group, possibly lysine, with a pK_{app} 9.4 was also involved (but see below). From more recent studies [26] it is now clear that such stable complexes only form when histidine-195 is protonated. The apparent dissociation constant for oxamate increases at 10-fold per unit of pH up to pH 11, that is 3.5 pH units above the apparent pK. Thus formation of the stable complex involves obligatory protonation of the histidine and one mole of H^+ is taken up per mole of substrate bound at high pH (Holbrook, unpublished result) (scheme 2).

3. The kinetics of enzyme–nucleotide reactions

Detailed kinetic investigation of nucleotide association and dissociation rates can give valuable information complementing that obtained from equilibrium binding experiments. The interaction of NADH with H_4LDH has been studied by a wide range of methods and can be used to highlight both the problems involved and the interpretation required in such investigations. The comprehensive steady state kinetic analysis of the bovine H_4LDH system in Schwert's laboratory [32] provided estimates of $2 \times 10^7\ M^{-1}\ sec^{-1}$ and $100\ sec^{-1}$ for the association and dissociation, respectively, with NADH. Heck [37] studied the pig heart enzyme by observing the relaxation of NADH fluorescence in a temperature jump apparatus and obtained $4 \pm 1 \times 10^7\ M^{-1}\ sec^{-1}$ and $35 \pm 3\ sec^{-1}$ for the association and dissociation rates at pH 6 and 8. Kaplan et al. [38] recently reported that the record of protein fluorescence quenching during NADH binding represents a second order process while nucleotide fluores-

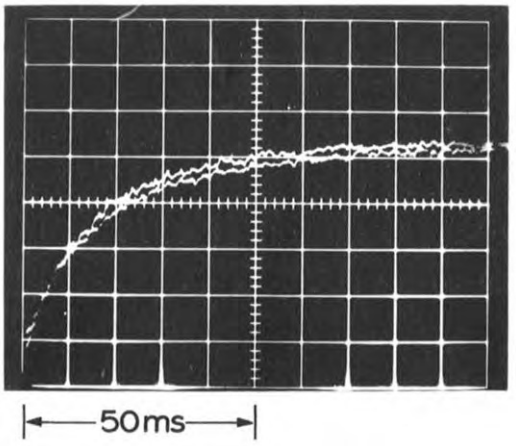


Fig. 2. The decrease in protein fluorescence after mixing a 0.4 μM (in sites) solution of H_4 lactate dehydrogenase with a 2.4 μM solution of NADH containing 5 mM oxamate (in 0.067 M phosphate buffer, pH 7.2) at 20°. In the reaction represented by the lower trace both solutions also contained 10% (by volume) ethanol. The dead-time of the stopped-flow fluorimeter is approx. 1.5 msec. The start of the reaction (full protein fluorescence) is one large division below the beginning of the frame.

cence enhancements result in a record of a first order reaction. This interpretation is, however, not valid. It has been pointed out by Gutfreund [39] that a reversible process E + NADH $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ ENADH (with k_1 a second order rate constant and k_{-1} a first order rate constant) proceeds towards equilibrium as an apparently first order reaction if the concentrations are such that the reaction reaches less than 80% completion. Under similar conditions the non-linear response of protein fluorescence quenching [13] will give an apparent second order reaction profile. In fact stopped-flow measurements of both types of fluorescence signal agree with the assignment of $k_1 = 5 \times 10^7\ M^{-1}\ sec^{-1}$ and $k_{-1} = 50 \pm 10\ sec^{-1}$. The dissociation constants calculated from kinetic and equilibrium measurements are in remarkably good agreement considering the difficulties of preparing really pure NADH and the different effects trace impurities have on different measurements. These results virtually exclude the possibilities of either two step reactions or site interaction in the association of NADH with pig heart LDH.

Fig. 2 shows the records of two observations of the quenching of protein fluorescence when solutions of H_4LDH (0.4 μM in sites) were mixed with solutions of 2.4 μM NADH and 5 mM oxamate, both in 67 mM phosphate buffer pH 7.2. The addition of oxamate results in the rapid formation of $E^{NADH}_{oxamate}$ from the ENADH complex. In this way there is complete conversion of enzyme sites to the ternary complex with the association of E and NADH as the rate determining step. In one of the two experiments both solutions also contained 10% v/v ethanol. The conditions of these experiments result in a pseudo first order plot if the corrections for non-linearity of protein fluorescence are carried out. This clearly shows that the association rate is not affected by the presence of ethanol, while Stinson and Holbrook [10] have demonstrated that the addition of 10% ethanol gives a threefold increase in the dissociation constant and only a 15% increase of the steady state rate of lactate oxidation (Holbrook unpublished). The rate of dissociation of NADH from the binary complex must increase threefold on addition of ethanol. One can thus conclude from these results that the dissociation of NADH is not rate limiting for the overall reaction.

4. Transients and single turnover reactions

The methods available for following and analysing the reactions of an enzyme during the transient phase between mixing with substrates and the attainment of the steady state have been recently reviewed by one of us [40]. The following transient experiments have given information about the mechanism of NAD^+ linked dehydrogenases.

The rate of formation of NAD(P)H during the pre-steady state phase was first observed with glutamate dehydrogenase [41]. In the case of liver alcohol dehydrogenase isotopic replacement of hydrogen by deuterium in ethanol and the reduced nucleotide was first used by Shore and Gutfreund [42] to locate the hydride transfer step. The transient formation of enzyme bound NADH during the reaction

$$E^{NAD^+}_{EtOH} \overset{slow}{\rightleftharpoons} E^{NADH}_{aldehyde} + H^+$$

(both NAD^+ and ethanol at saturating concentration)

$$E^{NADH} \xrightarrow{slow} E + NADH \quad (\text{fast})$$

is represented by a single exponential with an amplitude equivalent to the number of enzyme sites in solution. This indicates that both sites of the dimeric en-

zyme are kinetically equivalent in this reaction. If deuterated ethanol is used as the substrate the rate of formation of enzyme bound NADH is slower by a factor of 6 [42]. This indicates that the hydride transfer reaction is rate limiting for this process. The dissociation of aldehyde from $E^{NADH}_{aldehyde}$ will therefore be fast compared with the chemical reaction and this prevents any equilibration of the ternary complexes.

Studies of the transient formation of enzyme bound NADH from $E^{NAD^+}_{lact}$ with H_4 lactate dehydrogenase show some interesting differences in the kinetic behaviour of this enzyme compared with liver alcohol dehydrogenase. The transient has two rate components when observed by absorbancy at 340 nm. First there occurs a very rapid (within 1 msec) formation of NADH, which corresponds to 11% of the enzyme sites at pH 6, 20% of sites at pH 7 and 33% of the sites at pH 8, and subsequently there occurs a transient (275 sec^{-1} at pH 8) with an amplitude such that all sites are occupied with NADH when the steady state is reached. The observable transient rate is not affected significantly when deuterated lactate is used as a substrate. The conclusion from these observations is that the rapid step is due to the equilibration:

$$\overset{*}{E}B{:}\begin{matrix}\diagup NAD^+\\ \diagdown lact\end{matrix} \rightleftharpoons \overset{*}{E}BH^+\begin{matrix}\diagup NADH\\ \diagdown pyruvate\end{matrix}$$

and the measured transient rate is due to the dissociation of pyruvate to form ENADH prior to the dissociation of NADH, the latter reaction being rate limiting for the steady state. These postulates are further supported by observations described in the legend of fig. 3.

The relative fluorescence (excited at 340 nm, emitted at 430 nm) of NADH, H_4LDH bound NADH and NADH in the ternary complex $EBH^+\begin{matrix}\diagup NADH\\ \diagdown oxamate\end{matrix}$ is 1, 3.5 and 0.3. The rapid phase of the transient observed in spectrophotometric observation is reduced to a very small effect when nucleotide fluorescence is observed. This can be explained by the supposition that the rapid phase is due to the formation of $EBH^+\begin{matrix}\diagup NADH\\ \diagdown pyruvate\end{matrix}$ which has a very low fluorescence yield like $EBH^+\begin{matrix}\diagup NADH\\ \diagdown oxamate\end{matrix}$. The transient formation of enzyme bound NADH in the H_4 lactate dehydrogenase reaction was also monitored through the resulting quenching of protein fluorescence. These experiments were in agreement with the spectrophotometric observation that, at pH 7, 20% of the enzyme sites are converted into $EBH^+\begin{matrix}\diagup NADH\\ \diagdown pyruvate\end{matrix}$ while the subsequent observable rate corresponds to the formation of ENADH. The detailed analysis of the rates and amplitudes of the transient changes in nucleotide and protein fluorescence are being studied at present.

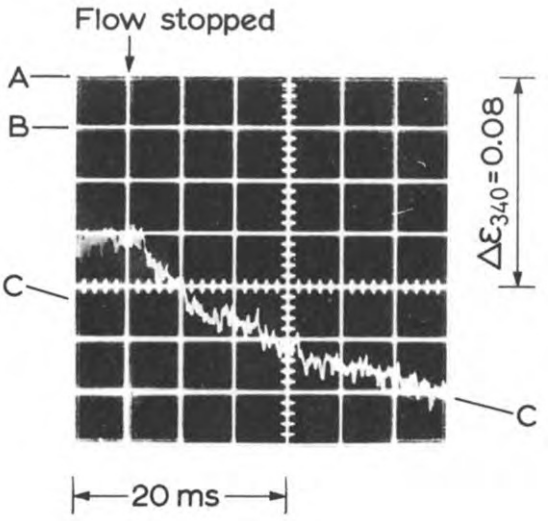


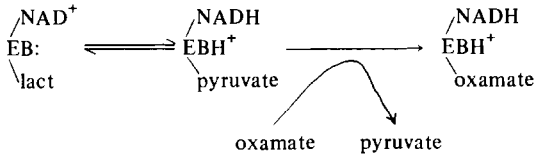
Fig. 3. The increase in extinction at 340 nm recorded in a split-beam stopped-flow spectrophotometer when a solution of 30 μM (in sites) of H_4 lactate dehydrogenase was mixed with a solution containing 13 mM NAD^+ and 100 mM L-lithium lactate (0.1 M phosphate, pH 7.0). The first order rate of approach to the steady state rate (line C) was 180 sec^{-1}. This transient extrapolated back 3 msec (age of reaction mixture in observation chamber when flow stopped) goes to line B on the $\Delta\epsilon_{340}$ scale. The change from line A($\Delta\epsilon_{340}$ = 0) to line B is due to a much faster change in extinction than the observed transient.

In the case of many hydrolytic reactions valuable information came from single turnover experiments with enzyme concentrations equal to or larger than the substrate concentration and the substrate dissociation constant [43]. In the case of dehydrogenases interesting results were obtained when ENADH was reacted with excess substrate. Experiments of the kind reported for liver alcohol dehydrogenase [42]

and M_4 lactate dehydrogenase [44] were also carried out with H_4 lactate dehydrogenase. The progress of the reactions of the complex 10 μM enzyme sites – 9 μM NADH and the complex 30 μM enzyme sites – 9 μM NADH with excess pyruvate was compared and each was found to be represented by a single exponential of identical rate and amplitude. This rate is equal to, and therefore rate limiting for, the enzyme steady state turnover. This again leads to the conclusion that the tetrameric enzyme has four identical and independent sites. When the deuterium analogue of NADH was used for such experiments the rate of the reaction was 90% of normal and thus a rate limiting isomerisation of the rapidly formed ternary complex with pyruvate must therefore occur before the hydride transfer.

5. Proton release and uptake

When the release of protons is monitored by carrying out the reaction of H_4 lactate dehydrogenase with lactate and NAD^+ at pH 8 in the presence of phenol red or at pH 6 in the presence of chlorophenol red, the transient ($k = 275\ sec^{-1}$) and the steady state rates are observed. There is, however, no instant proton release corresponding to the instant formation of NADH ascribed to the rapid equilibration of the ternary complexes. If oxamate is added to the solution of lactate and NAD^+ before mixing with enzyme, the transient formation of enzyme bound NADH, monitored at 340 nm, is much more clearly separated from the steady state. Oxamate combines rapidly with ENADH to form an inhibitor ternary complex and the rate of formation of this complex is determined by the rate of formation of ENADH. If proton release is monitored with a reaction mixture containing oxamate no transient is observed. From these observations it was concluded that there is conservation of charge during interconversion of ternary complexes: the stable reduced ternary complex with oxamate (or pyruvate) is always protonated.



Mixing ENADH with oxamate at pH 8 results in an uptake of protons from the medium which is too fast for rate analysis by the stopped-flow technique. While at pH 8 the amplitude of the fast process corresponds to one mole of protons taken up per mole of oxamate bound, at pH 6 only 0.2 equivalent of proton uptake per mole of oxamate bound was observed, as expected for a histidine of pK 6.7 [68].

Temperature relaxation experiments were carried out on a reaction mixture of H_4 lactate dehydrogenase, NADH and oxamate in the presence of the respective indicator at pH 8 and 6. The amplitude of the relaxation was dependent on oxamate concentration. In the absence of oxamate no relaxation was observed on the time scale of fig. 4. As the oxamate concentration was increased a maximum amplitude was reached and this is the condition of the experiments recorded in fig. 4. The relaxation time was independent of oxamate concentration at this high enzyme concentration. The very much smaller amplitude of proton release during temperature relaxation at pH 6 as compared with pH 8 is in agreement with other experiments indicating that oxamate binding to ENADH is linked to the protonation of a group on the enzyme which has a pK of 6.7.

5.1. *o-Nitrophenylpyruvate*

Poor substrates (low V_{max}) of a suitable kind (reasonable K_m) can be useful in resolving elementary steps in a mechanism since they can allow the concentrations of intermediates to build up to levels unattainable when the catalytic step is rapid. Nitrophenylpyruvate is reduced by lactate dehydrogenase at a maximum rate 1/2000th of that of pyruvate. This rate is not markedly pH dependent. The substrate rapidly forms a ternary complex with enzyme and NADH which can be detected by the reduction in the enhanced fluorescence of bound NADH which is complete before the much slower conversion of NADH to NAD^+. Since the subsequent slow steady state does not show an isotope effect with NADD nor a rapid transient formation of the first mole of products, the rate limiting step is the isomerisation of the initially formed ternary complex, exactly as with pyruvate as substrate. The true dissociation constant of nitrophenylpyruvate shows exactly the same pH dependence as the dissociation constant for oxamate (fig. 5) and thus there is compulsory uptake of the

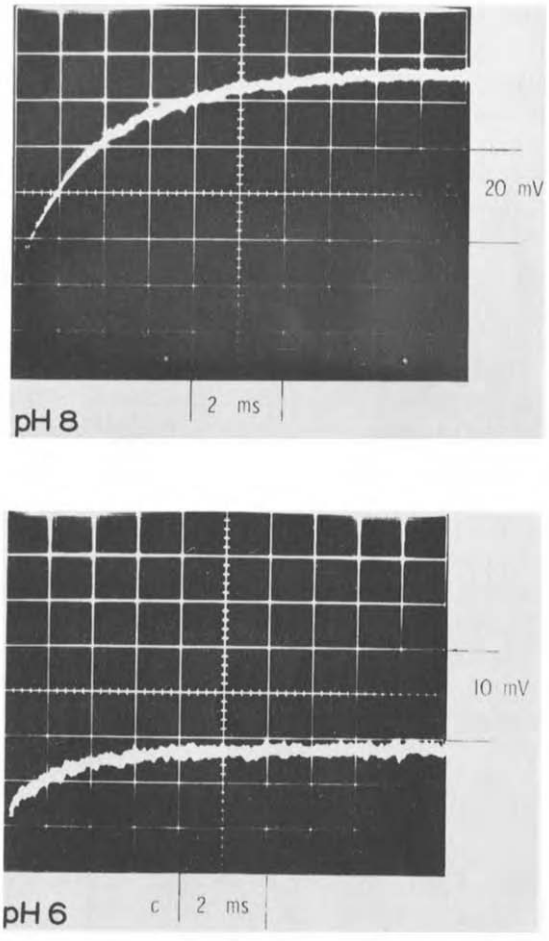


Fig. 4. Temperature jump displacement of oxamate from the complex $E^{NADH}_{oxamate}$ (36 μM in sites) results in a concomitant liberation of protons. This was monitored at pH 8 (upper trace) at 560 nm in the presence of phenol red and at pH 6 (lower trace) at 580 nm in the presence of chlorophenol red. In both cases solution was 0.1 M in NaCl and the indicator concentration was adjusted to give approximately the same change in transmission for the equivalent liberation of protons. Both experiments were carried out at oxamate concentrations giving the maximum relaxation amplitude (90 μM oxamate at pH 8 and 45 μM oxamate at pH 6).

proton with this substrate either before or rapidly after the binding of the substrate but before the rate limiting isomerisation [26]. The proton uptake can be observed with a glass electrode (Holbrook, unpublished).

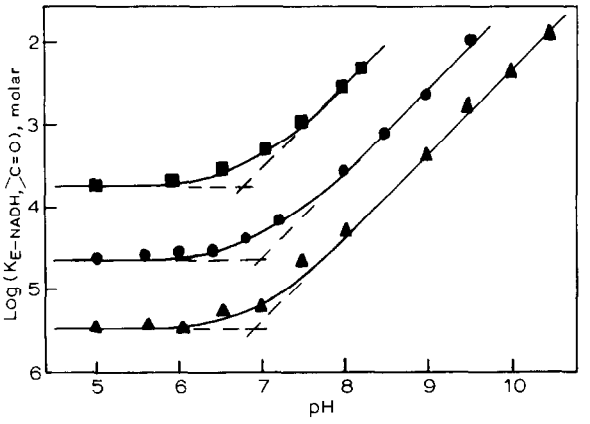


Fig. 5. Ternary complexes of NADH are protonated. The apparent dissociation constant of nitrophenylpyruvate (■) and oxamate (▲) from the E-NADH complex of H_4 LDH and of oxamate from M_4 LDH (●) increases 10-fold per unit of pH above an apparent pK of 6.8. A model is:

$$EB: \quad {}^{NADH}\!/ \;\; pK_a = 6.8 \;\; {}^{NADH}\!/ \rightleftharpoons EBH^+ + \rangle C{=}O \;\; \overset{K_d}{\rightleftharpoons} \;\; {}^{NADH}\!/ \; EBH^+ \; \backslash C{=}O$$

$$K_{app} = K_d\left(1 + \frac{K_a}{[H^+]}\right)$$

6. Chemistry of binding and catalysis

Horse liver alcohol dehydrogenase is a dimer of two identical subunits of molecular weight 40,000 [45, 46]. The dimer contains 4 mole Zn^{2+}. Two of the four zinc atoms are important to stabilise the enzyme but can be removed to leave an active although unstable protein [47, 48]. One cysteine residue in each subunit reacts rapidly with iodoacetic acid with loss of enzyme activity [49]. The carboxymethylated enzyme can still bind NADH and retains its zinc [50]. Modification of the lysine residues of alcohol dehydrogenase with methylpicolinamidate activates the enzyme 15-fold. In the enzyme with modified lysines, the binding of NADH becomes much weaker, resulting in a faster steady state rate [51] because NADH dissociation is normally rate limiting. Activation of the enzyme also occurs after treatment with diethylpyrocarbonate [52], possibly for the same reason.

Tryptophan residues were implicated in the mechanism of many dehydrogenases by Schellenberg and his coworkers. However, in the case of lactate dehy-

drogenase [53] the work could not be repeated [54, 55] and there is increasing evidence against the participation of tryptophan in other dehydrogenases as well [56]. Large changes in tryptophan fluorescence on binding NADH are due to energy transfer and it is not necessary to postulate direct interaction with tryptophan [13].

From the early report by Kubovitz and Ott [27] that lactate dehydrogenase could be crystallised as an inactive mercuric salt and reactivated by thiols there has been much interest in the role of cysteine residues in the activity of the enzyme. By about 1967 it was apparent [57–59] that all lactate dehydrogenases contain one cysteine residue in a highly conserved sequence of amino acids and that blocking this residue specifically inhibited the enzyme. Blocking the essential thiol has no effect on the ability of the enzyme to bind coenzyme [60] but completely abolishes the binding of substrates [61]. Inspection of the three-dimensional structure of the dogfish enzyme shows that the essential thiol is at the centre of the subunit in a region which might be expected to have a highly conserved sequence, but is too far from the substrate binding site to allow direct interaction [62]. Any modification of this residue would be expected to alter the environment of the essential histidine residue.

It was speculated that the group with apparent pK of 7 involved in binding substrate to LDH, was a histidine and that it acted as a source and a sink of the H^+ in the dehydrogenase reaction [32, 35, 36]. Photo-oxidation studies did not conclusively implicate a histidine [63]. Alkylation experiments were usually inconclusive but Jeckel and Pfleiderer [64] developed the use of $HgCl_2$ to protect the thiol reversibly. Woenckhaus and his coworkers were then able to show that the inhibition of lactate dehydrogenase by a 3-bromoacetylpyridine was due to alkylation of a histidine and found only a little radioactivity in the essential thiol peptide [65]. From experiments with bromopyruvate and NAD^+ it is clear that the essential histidine is associated with substrate binding [66, 67]. Similar histidine peptides are present in all lactate dehydrogenases and can be fitted to the electron density map at the active site of the dogfish enzyme. Based on the dogfish backbone numbering sequence the residue is histidine-195. Using chemical modifications [68] it has recently been shown that histidine-195 has a pK of 6.8 and that the pK_{app} of 6.8 seen in the kinetics and binding of substrates is due to ionisations of this residue. Direct identification of an apparent pK with the pK of an individual group is not normally possible. The imidazole part of histidine-195 reacts with diethylpyrocarbonate and the reaction can be followed by the appearance of the ultraviolet spectrum of carbethoxyhistidine (fig. 6). The apparent pK for the reaction is 6.8. Normally this apparent pK could not be ascribed to the individual histidine because it might reflect the pK of groups which control access of the inhibitor to the histidine. However, the pig heart enzyme histidine reacts 10 times faster than free histidine and thus the rate limiting step cannot be access of the inhibitor. The reaction is first order in diethylpyrocarbonate and this suggests that the reagent does not bind prior to acylation of the histidine [69]. The enzyme histidine is properly described as reactive since it has the same pK as imidazole and N^{α}-acetylhistidine but a 10 times greater rate of reaction than the free imidazoles. Addition of one mole of inhibitor produces one mole of carbethoxylated enzyme histidine and a total loss of enzyme activity. The reactivity of the histidine is only reduced by half in the E^{NADH} complex and the pK is still about 6.7 [68]. Rapid equilibrium fluorescence titration equipment [10] was used to show that NADH binds normally to the carbethoxylated enzyme but that the substrate analogue oxamate and the substrate nitrophenylpyruvate can no longer bind. Thus the histidine which reacts with diethylpyrocarbonate is at the substrate binding site and equivalent to the histidine-195 identified earlier by Woenckhaus [66, 67]. Formation of a ternary complex of the enzyme with NADH and oxamate completely protected the histidine from reaction with diethylpyrocarbonate. Now since protonated imidazoles do not react with the acylating agent and it is known that the ternary complex is always protonated with an apparent pK of 6.8, the identification of the apparent pK of substrate binding with the pK of histidine-195 is complete [26, 68].

It has been suggested that a lysine group with pK = 9.4 might be involved in binding substrates to LDH [35]. No such pK was found in a more recent re-examination of oxamate binding in the presence of high enough concentrations of NADH to keep the enzyme saturated with this nucleotide at high pH [26], and it is unlikely that lysine residues are in-

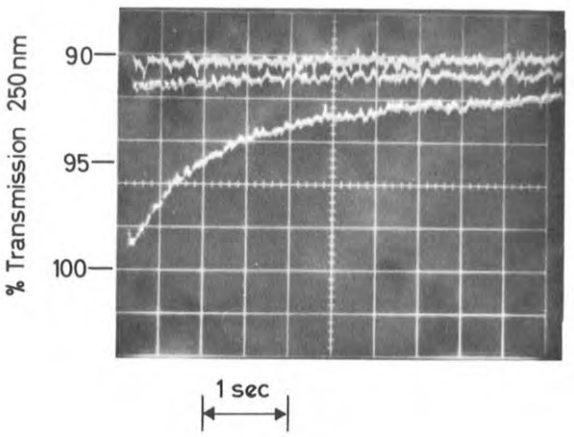


Fig. 6. Rapid reaction of the essential histidine of pig heart LDH with diethylpyrocarbonate. The photograph is of the stopped flow trace of the change in transmission of light at 250 nm in a stopped-flow apparatus after the rapid mixing of equal volumes of LDH (26 μM subunits) and diethylpyrocarbonate (10.6 mM) at pH 8. The lower trace was triggered as flow stopped. The upper two traces immediately follow the first. The decrease in transmission is due to the formation of carbethoxyhistidine which absorbs at 240 and 250 nm. The amplitude of the rapid phase corresponds to the formation of one mole of carbethoxyhistidine per mole subunits. The rate ($200\ \ell\ mol^{-1}\ sec^{-1}$) with the essential enzyme histidine is 10-fold faster than with free histidine or imidazole of the same pK at the same pH.

volved in binding [70, 71]. There is, however, general agreement that the binding of NADH becomes very weak above pH 9. Several attempts have been made to identify the groups involved. Neither lysines [71] nor the essential thiol group [61] are involved. Nitration of tyrosines does lead to changes in the K_m for NAD^+ [72].

7. Site equivalence

In recent years a variety of phenomena observed during reactions of polymeric enzymes with substrates, substrate analogues and reagents have been interpreted variously as asymmetry, half of site reactivity or negative cooperativity. The methods for the study of the individual steps in the reactions of NAD linked dehydrogenases, which are discussed in this review, are ideally suitable for a quantitative evaluation of any deviation from the independent action and equivalence of different sites on one enzyme molecule. It should be made clear at the outset that any attempt to generalise about the behaviour of even such a relatively small portion of enzymes as that represented by dehydrogenases is doomed to failure. The examples are intended to illustrate methods of investigating site equivalence rather than to make definitive statements about these enzymes under all possible conditions.

In principle the easiest phenomenon to investigate is the binding of reduced nucleotides to enzymes. Large signals are obtained from nucleotide and protein fluorescence and often quite respectable extinction changes occur during the complex formation [5]. Yet the subject has been bedevilled with artifacts [73]. Careful studies of NADH binding to H_4 and M_4 lactate dehydrogenases and liver alcohol dehydrogenase have shown that the multiple binding sites are completely equivalent and identical. For NAD binding the experimental techniques are more difficult; so much so that some of the phenomena ascribed to NAD binding (quenching of lactate dehydrogenase fluorescence) are due only to the impurities in the nucleotide. In the cases of beef liver glutamate dehydrogenase and various glyceraldehyde 3-phosphate dehydrogenases real non equivalence and real cooperativity of nucleotide binding sites have been observed but not yet unambiguously explained.

The transient formation of enzyme bound NADH and the oxidation of stoichiometric ENADH complexes by mixing with excess substrate have both been used to investigate the kinetic equivalence of the four sites in the two lactate dehydrogenases and the two sites of liver alcohol dehydrogenase (see sect. 3 and 4). Since there are several reports in the literature [74] which are in convlict with our conclusion that the three enzymes discussed here have thermodynamically and kinetically independent sites, some effects which give apparent non equivalence must be discussed. The transient or single turnover can be described as the sum of a series of exponentials. In the case of independent sites the number of exponentials involved will depend on the number of steps in the mechanism and their rates and equilibria. In the particular case of NAD^+ linked dehydrogenases each reaction species containing NADH can be responsible for a distinct phase in the spectral or fluorescence signal observed. The observation of extinction changes

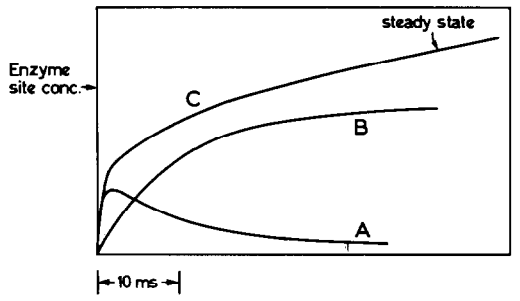


Fig. 7. Analogue computer simulation of the progress of a hypothetical reaction

$$E + NAD^+ + RHCOH \rightleftharpoons E^{NAD^+}_{RHCOH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^{NADH}_{RC=O} + H^+$$

$$E^{NADH}_{RC=O} \xrightarrow{k_2} E^{NADH} \xrightarrow{k_3} E + NADH$$

$k_1 = 1000\ sec^{-1}$, $k_{-1} = 1000\ sec^{-1}$, $k_2 = 200\ sec^{-1}$, $k_3 = 10\ sec^{-1}$.

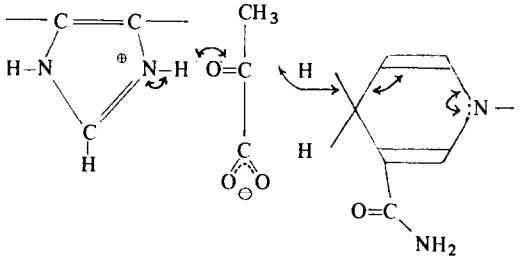
The reactant concentrations are high enough for the rate of formation of $E^{NAD^+}_{RHCOH}$ to be much faster than any of the other steps. Experimental observation at 340 nm would result in a triphasic record (two transient phases and the steady state) as represented by line C. Line A shows the record of formation and decomposition for $E^{NADH}_{RC=O}$ and line B the record for the formation of E^{NADH}.

at 340 nm as a monitor of the appearance of NADH (enzyme bound and free) during the reaction of H_4 LDH with lactate and NAD^+ showed two transient phases. The evidence presented here indicates that the two transient phases are due to rapid equilibration of the ternary complexes and a step involved in the release of pyruvate. It is likely that in other systems the appearance of two transient phases is also due to two distinct steps in the mechanism rather than to different reaction rates for individual sites. An analogue computer simulation of such a system is presented and explained in fig. 7.

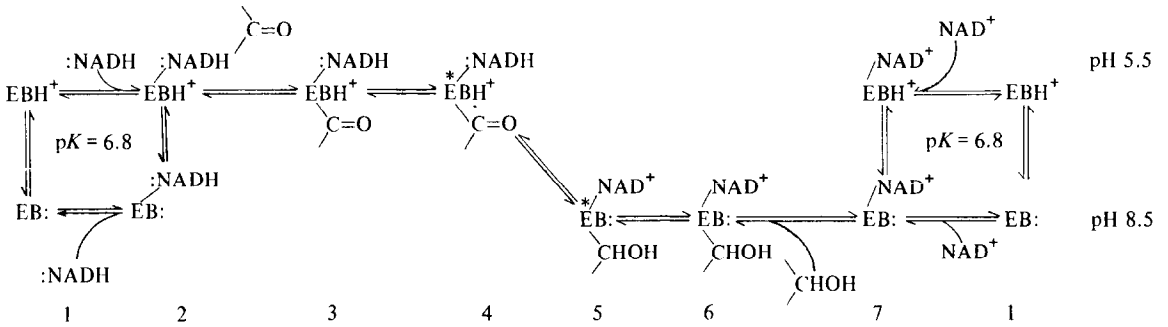
The appearance of asymmetric reaction profiles in the single turnover of a polymeric enzyme is sometimes found when slowly reacting substrate analogues [75] or reagents [76] are used to explore active sites. This is likely to be due to the transmission of substrate induced conformation changes from one subunit to another during the relatively long period between the reaction of successive units within one enzyme molecule.

7.1. *A mechanism for lactate dehydrogenase*

The object of this review has been to present the methods which are available to investigate the nature of intermediates in dehydrogenase catalysis. While the methods are now established they have not yet been applied to any enzyme over a wide enough pH range to enable a definitive mechanism to be presented. Scheme 3 is the simplest we can devise to explain the spectral and fluorescence properties of the intermediates, the pH dependencies of their equilibria and rates of interconversion and, not least, the pH dependence of K_m and V_{max} in both directions. It is the basis on which experiments are planned and it is suggested that it will be valuable to reexamine the pH-dependence of V_{max} and K_m for lactate oxidation at low pH with pure substrates. The scheme does, however, show how a protonated histidine is brought close to pyruvate and NADH so that the rapid catalytic equilibrium (4 ⇿ 5) need only involve the electron transfer from NADH and the proton transfer from histidine envisaged by Schwert as long ago as 1959 [36]:



The more recent experiments from these laboratories have provided identification of the ionisation state of the particular histidine in the mechanism. The steps at which changes in protonation of this histidine occur have been placed in the sequence of events in the reaction of pig heart lactate dehydrogenase with its substrates. Direct study of the points at which protons are liberated leads us to emphasise the charge conservation within the ternary complexes of lactate dehydrogenase. Attention is also drawn to the interesting result that the position of the equilibrium for the rapidly interconverting ternary complexes can be observed. Perhaps the more important tasks for



Scheme 3. A simple mechanism for lactate dehydrogenase. The base B: is the imidazole of histidine-195. Pyruvate is $\succ C{=}O$ and lactate is $\succ CHOH$. Forms shown as $\overset{*}{E}$ are isomers of unknown structure, but different from those shown E. The pK of the histidine in the free enzyme, and in binary complexes with NAD^+ and NADH is 6.8. Thermodynamically stable ternary complexes with NADH are protonated. Ternary complexes with NAD^+ and lactate are unprotonated although the evidence only rests upon the K_m for lactate. The scheme emphasises isomerisation of ternary complexes, charge conservation of ternary complexes and 'compulsory protonation' of ternary complexes with NADH. Evidence is given in the text that the step 3 → 4 is the transient in pyruvate reduction. During the oxidation of lactate the measurable transient rate of production of NADH and the steady state rate are steps between complex 4 and complex 2. A more complex scheme including alternate pathways is of course not excluded but the steps shown here are sufficient to accommodate the available experimental data. The scheme explains how one mole of NADH can be produced when E-NAD^+ is mixed with lactate and oxamate at pH 8.5 without liberation of a proton. Between complex 7 and protonated complex 2 there is no liberation of H^+ to the solvent. In the presence of enough oxamate, the compulsorily protonated complex EBH^+–NADH–oxamate will form before complex 2 can lose its proton to the solvent or lose NADH. A reduced abortive ternary complex forms with high lactate at low pH. With pure substrates, the V_{max} in both directions should not be dependent upon pH. This is experimentally found for pyruvate reduction and for lactate oxidation from pH 10 to at least pH 7.2. The spectral and fluorescence properties of the various complexes are described in the text.

the future are to discover a structural basis for the compulsory protonation of the ternary complexes involving NADH and to relate the kinetically defined isomerisations to the known conformations of stable ternary complexes which can be defined crystallographically.

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References

[1] H. Sund and H. Theorell, The Enzymes 7 (1963) 25–83.
[2] G.W. Schwert and A.D. Winer, The Enzymes 7 (1963) 127–148.
[3] Y. Takenaka and G. Schwert, J. Biol. Chem. 223 (1956) 157–170.
[4] W.B. Novoa and G.W. Schwert, J. Biol. Chem. 236 (1961) 2150–2153.
[5] C.H. McMurray, H.d'A Heck and H. Gutfreund, Biochem. J. 108 (1968) 793–796.
[6] K. Dalziel, J. Biol. Chem. 238 (1963) 2850–2858.
[7] H. Theorell and R. Bonnichsen, Acta Chem. Scand. 5 (1951) 1105–1126.
[8] B. Chance and J.B. Neilands, J. Biol. Chem. 199 (1952) 383–387.
[9] O. Warburg and W. Christian, Biochem. Z. 287 (1936) 291–328.
[10] R.A. Stinson and J.J. Holbrook, Biochem. J. 131 (1973) 719–728.
[11] E. Silverstein and P.D. Boyer, Biochemical Preparations 11, 89–95.
[12] T. Yonetani and H. Theorell, Arch. Biochem. Biophys. 99 (1962) 433–446.
[13] J.J. Holbrook, Biochem. J 128 (1972) 921–931.
[14] H. Theorell and J.S. McKinley-McKee, Acta Chem. Scand. 15 (1961) 1811–1833.
[15] S.R. Anderson and G. Weber, Biochemistry 4 (1965) 1948–1957.
[16] A.D. Winer, Acta Chem. Scand. 17 (1963) s203–s209.
[17] H. Theorell and A.D. Winer, Arch. Biochem. Biophys. 83 (1959) 291–308.

[18] Th. Förster, Discuss. Faraday Soc. 27 (1959) 7–17.
[19] S.F. Velick, J. Biol. Chem. 233 (1958) 1455–1467.
[20] R.H. McKay and N.O. Kaplan, Biochem. Biophys. Acta 79 (1964) 273–283.
[21] H. Theorell and K. Tatemoto, Arch. Biochem. Biophys. 142 (1971) 69–82.
[22] J.J. Holbrook, Biochem. J. 128 (1972) 921–931.
[23] J.J. Holbrook, D.W. Yates, S.J. Reynolds, R.W. Evans, C. Greenwood and M.G. Gore, Biochem. J. 128 (1972) 933–940.
[24] G. Karreman, R.H. Steele and A. Szent-Gyorgyi, Proc. Natl. Acad. Sci. U.S. 44 (1958) 140–143.
[25] S.P. Colowick, J. van Eys and J.H. Park, Comprehensive Biochemistry 14 (1966) 1–98.
[26] J.J. Holbrook and R.A. Stinson, Biochem. J. 131 (1973) 739–748.
[27] F. Kubowitz and P. Ott, Biochem. Z. 314 (1943) 94–117.
[28] H.J. Fromm, Biochim. Biophys. Acta 52 (1961) 199–200.
[29] G. di Sabato, Biochem. Biophys. Res. Commun. 33 (1968) 688–695.
[30] H. Gutfreund, R. Cantwell, C.H. McMurray, R.S. Criddle and G. Hathaway, Biochem. J. 106 (1968) 683–687.
[31] A.D. Winer, W.B. Novoa and G.W. Schwert, J. Am. Chem. Soc. 79 (1957) 6571–6572.
[32] G.W. Schwert, B.R. Millar and Peanasky, J. Biol. Chem. 242 (1967) 3245–3252.
[33] G. Pfleiderer, D. Jeckel and Th. Wieland, Biochem. Z. 328 (1956) 187–194.
[34] H. Theorell and T. Yonetani, Biochem. Z. 338 (1963) 537–553.
[35] A.D. Winer and G.W. Schwert, J. Biol. Chem. 234 (1959) 1155–1161.
[36] W.B. Novoa, A.D. Winer, A.J. Glaid and G.W. Schwert, J. Biol. Chem. 234 (1959) 1143–1148.
[37] H.d'A. Heck, J. Biol. Chem. 244 (1969) 4375–4381.
[38] J. Everse, R.L. Berger and N.O. Kaplan, in: Structure and function of oxidation reduction enzymes, eds. Å. Åkeson and Å. Ehrenberg (Pergamon Press, Oxford and New York, 1972) p. 691.
[39] H. Gutfreund, Enzymes: physical principles (Wiley, London, New York, 1972) p. 122.
[40] H. Gutfreund, Ann. Rev. Biochem. 40 (1971) 315–344.
[41] M. Iwatsubo and D. Pantaloni, Bull. Soc. Chim. Biol. Paris, 49 (1967) 1563–1572.
[42] J.D. Shore and H. Gutfreund, Biochemistry 9 (1970) 4655–4659.
[43] T.E. Barman and H. Gutfreund, Biochem. J. 101 (1966) 411–416.
[44] R.A. Stinson and H. Gutfreund, Biochem. J. 121 (1971) 235–240.
[45] H. Jörnvall, European J. Biochem. 16 (1970) 25–40.
[46] H. Jörnvall, European J. Biochem. 16 (1970) 41–49.
[47] H. Weiner, Biochemistry 8 (1969) 526–533.
[48] I. Iweibo and H. Weiner, Biochemistry 11 (1972) 1003–1010.
[49] I. Harris, Nature 203 (1964) 30–34.
[50] Ting-Kai Li and B. Vallee, Biochemistry 4 (1965) 1195–1202.
[51] B.V. Plapp, J. Biol. Chem. 245 (1970) 1727–1735.
[52] D.L. Morris and J.S. McKinely-McKee, European J. Biochem. 29 (1972) 515–520.
[53] K.A. Schellenberg, J. Biol. Chem. 242 (1967) 1815–1820.
[54] J.J. Holbrook, P.A. Roberts, B. Robson and R.A. Stinson, Abst. Int. Cong. Biochem. 8th (1970) 83–84.
[55] W.S. Allison, H.B. White and M.J. Connors, Biochemistry 10 (1971) 2290–2296.
[56] L.M. Allen and R.G. Wolfe, Biochem. Biophys. Research Commun. 41 (1970) 1518–1522.
[57] J.J. Holbrook and G. Pfleiderer, Biochem. Z. 342 (1965) 111–114.
[58] T.P. Fondy, J. Everse, G. Driscoll, F. Castillo, F.E. Stolzenbach and N.O. Kaplan, J. Biol. Chem. 240 (1965) 4219–4234.
[59] J.J. Holbrook, G. Pfleiderer, K. Mella, M. Volz, W. Leskowac and R. Jeckel, European J. Biochem. 1 (1967) 476–481.
[60] J.J. Holbrook, Biochem. Z. 244 (1966) 141–152.
[61] J.J. Holbrook and R.A. Stinson, Biochem. J. 120 (1970) 289–297.
[62] M.J. Adams, A. McPherson, M.G. Rossmann, R.W. Schevitz, I. Smiley and A.J. Wonacott, in: Pyridine nucleotide-dependent dehydrogenases, ed. H. Sund (Springer Verlag, Berlin, 1970) p. 157–171.
[63] D.B. Millar and G.W. Schwert, J. Biol. Chem. 238 (1963) 3249–3255.
[64] D. Jeckel and G. Pfleiderer, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 903–914.
[65] C. Woenckhaus, J. Berghauser and G. Pfleiderer, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 473–483.
[66] J. Berghauser, I. Falderbaum and C. Woenckhaus, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 52–58.
[67] C. Woenckhaus, E. Schattle, R. Jeck and J. Berghauser, Hoppe-Seyler's Z. Physiol. Chem. 353 (1972) 559–564.
[68] J.J. Holbrook and V.A. Ingram, Biochem. J. 131 (1973) 729–738.
[69] D.E. Schmidt and F.H. Westheimer, Biochemistry 10 (1971) 1249–1253.
[70] G. Pfleiderer, J.J. Holbrook, L. Zaki and D. Jeckel, FEBS Letters 1 (1968) 129–132.
[71] P.C. Yang and G.W. Schwert, J. Biol. Chem. 245 (1970) 4886–4893.
[72] D. Jeckel, R. Anders and G. Pfleiderer, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 769–779.
[73] P.L. Luisi and R. Favilla, European J. Biochem. 17 (1970) 91–94.
[74] S.A. Bernhard, M.F. Dunn, P.L. Luisi and P. Schack, Biochemistry 9 (1970) 185–192.
[75] A. Levitzki, W.B. Stallcup and D.E. Koshland, Biochemistry 10 (1971) 3371–3377.
[76] R.A. MacQuarrie and S.A. Bernhard, J. Mol. Biol. 55 (1971) 181–188.