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Liver Alcohol Dehydrogenase

III. Kinetics in the Presence of Caprate, *Iso*butyramide and Imidazole

HUGO THEORELL and JOHN S. McKINLEY-McKEE

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

1. The effects on the kinetics of the LADH-coenzyme-substrate system of caprate and *iso*butyramide at pH 7 are described. Caprate and *iso*butyramide have only inhibitory actions, which were localized to different parts of the reaction cycle and used for the calculations of various velocity constants. The results were in agreement with the equilibrium data from Part II.

2. Likewise, the effects of imidazole at pH 7 and 9 were studied in detail. It was found that imidazole could either increase (5 – 10 fold) or decrease the reaction velocity depending on the concentration of coenzymes and substrates. These effects were explained from the properties of the ternary enzyme-coenzyme-imidazole complexes.

3. It was found to be a general rule that the attachment of substrates or competing inhibitors to the substrate binding sites do not interfere appreciably with the "on" velocity constants of the coenzymes. The sometimes very large changes in dissociation constants, observed in equilibrium measurements, were parallelled by changes in the "off" velocity constants.

4. The mode of attachment of coenzymes, substrates and inhibitors is discussed. Zinc seems to be bound to the protein by three out of six octahedral bonds. The free bonds presumably hold water molecules, one of which at higher pH (pK 8.60) looses a proton. The resulting attraction between $Zn-OH^-$ and the positively charged pyridine ring of DPN is held responsible for the changes in $K_{E,O}$ with pH. This does not operate in the case of $K_{E,R}$ where the pyridine ring carries no charge.

5. It is concluded that in both ER and EO, the adenine moieties are attached to Zn by two bonds as third bidentate ligands, which as in the case of ethylene diamine are weak. Zinc is implicated as the center of a ternary complex with three bonds to the protein, two to the adenine and one to the substrates. In the binary complexes this latter bond is in EO joined to the pyridinium ring ($Zn-OH^--N^+$) but not in ER when it is occupied by a water molecule or at high pH a hydroxo group.

The two coenzymes select with great preference their partners by switching during interconversion to free one or the other of two out of three bonds, while each imposes by its different nature different properties on the third bond.

6. An abortive ternary complex ERalc formed at high alcohol concentrations is more stable than the binary ER complex. This

explains the inhibition of reaction velocity observed at high concentrations of alcohol in the LADH system.

7. Glycine like *ortho*phenanthroline and *aa'*-dipyridyl forms a binary complex with LADH but no ternary complexes including the coenzymes. It is assumed that glycine acts as a third bidentate ligand, excluding the adenine moieties of the coenzymes from being bound to the zinc.

It was earlier found that fatty acids form compounds of the type EI and EOI, but not ERI, and that fatty acid amides form compounds EI and ERI, but not EOI, and it was considered that the ternary complexes possessed a similar structure to the reactive complexes containing alcohol or aldehyde [1]. It was shown in Part II that imidazole and other nitrogenous compounds form compounds with both EO and ER. Whereas in the case of fatty acids and amides the ternary complexes are more stable than the binary ones, the opposite was found for imidazole. It was therefore of obvious interest to study the influence of these three different types of inhibitors on the kinetics in the hope of finding new facts on the nature of the binding sites for co-enzymes and substrates. Knowledge from Part II of the dissociation constants for imidazole provided an understanding of the stimulation rather than inhibition found with imidazole at high substrate concentrations, a phenomenon previously encountered with chloride [2].

Materials and methods were the same as those used in Parts I and II. In all but two of the sets of experiments the fluorometer rather than the spectrophotofluorometer was used due to greater sensitivity and stability.

RESULTS

It is customary to distinguish between three types of inhibition; competitive, noncompetitive and uncompetitive. In a system with two substrates, and the reaction occurring in two phases with alternative pathways, as is the case with alcohol dehydrogenase, the results do not always conform with any of these categories, and the interpretation of the experiments to be presented below therefore requires some comment.

Some inhibitors interfere only in the first reaction phase, and these have no influence on the break down of the complexes in the second phase. An interference of inhibitors with the breakdown of ternary complexes has never been observed and is not to be expected since the binding sites for coenzymes and substrates are already occupied. However, after the dissociation of the substrates, the binary EO (case 1 and 2, the "forward" reaction) and ER (case 3 and 4, the "reverse" reaction) can form EOI or ERI complexes which leads to a "second phase effect"; inhibition if the breakdown is retarded, as with caprate (EOI, Figs. 1 and 2) or *iso*butyramide (ERI, Figs. 7 and 8), or stimulation, if it is accellerated, as with imidazole (Figs. 11—13 b etc). These second phase effects appear near the intercepts with the ordinate, where both coenzyme and substrate are in high concentration, and the decomposition of the complexes in the second phase is more or less rate-limiting. When the inhibitor is unable to form ternary complexes with the second phase binary complexes, for

example *iso*butyramide with EO (case 1 and 2), or caprate with ER (case 3 and 4), no second phase effects occur. When both ERI and EOI can be formed, (as is the case with imidazole), second phase effects will always occur, but will not influence the reaction velocity in the special case when the decomposition rate of EO is the same as for EOI, as is not far from true with imidazole at pH 7 (case 1 and 2, Figs. 9a and 10.).

The second phase effects interfere only with the terms $1/k_2$ or $1/k_2'$ in the T.-C. formula (equations 1a and 1b, Part I), which are independent of coenzyme and substrate concentrations and changes in decomposition velocity by complex formation with inhibitors will therefore cause a parallel displacement of the Lineweaver-Burk plots upward (inhibition) or downward (stimulation). Thereby it may happen that plots result, which would normally be taken for un- or noncompetitive, or mixed *e.g.* Fig. 1. The correct interpretation, however, is in this case competitive first + uncompetitive second phase inhibition. It should be pointed out that unless equilibrium measurements have proved the formation of ternary complexes in the second phase, the distinction between the different inhibition types may be very difficult.

The relations between the types of inhibition in the T.-C. mechanism are determined by both rate and equilibrium constants in the following way. *Noncompetitive* means that the degree of inhibition is independent of the concentration of the varied reaction partner. The Lineweaver-Burk plots cross on the abscissa at a negative value for 1/[C]. An example of this is caprate, case 3 (Fig. 3). Caprate gives complexes with E (EI) and with EO (EOI), but not with ER. Second phase inhibition therefore does not occur and alcohol and caprate are strictly competitive (Fig. 4). In Fig. 3, at the intercept with the ordinate [DPN] = ∞, and every enzyme molecule, E, when liberated from the second phase reaction will immediately form EO that will either react with caprate (I) to form EOI, or with alcohol:

$$\mathrm{EOI} \rightleftharpoons \mathrm{EO} + \mathrm{alc} \xrightarrow{k_3'} \mathrm{ER} \xrightarrow{k_2'} \mathrm{E} + \mathrm{R} \qquad (1)$$

Since the k_3' and k_2' reactions are consecutive:

$$[\mathrm{EO}]\,[\mathrm{alc}]\,k_3' = [\mathrm{ER}]k_2' \qquad (2)$$

If [I] is chosen to give 50 % inhibition:

$$[\mathrm{EOI}] = [\mathrm{EO}] + [\mathrm{ER}] \qquad (3)$$

From (2) and (3): $[\mathrm{EO}]/[\mathrm{EOI}] = k_2/(k_2 + k_3\,[\mathrm{alc}])$ (4)

and $K_{\mathrm{EO,I}} = [\mathrm{I}]k_2/(k_2 + k_3'\,[\mathrm{alc}])$ (5)

Now the condition for noncompetitive, 50 % inhibition is that [alc] is such that half inhibition should also be found when [DPN]$\rightarrow$0, which requires:

$$[\mathrm{alc}]/[\mathrm{I}] = K_{\mathrm{E,alc}}/K_{\mathrm{EI}} \qquad (6)$$

Combining (5) and (6) gives:

$$\frac{1}{[\mathrm{alc}]} = \frac{K_{\mathrm{EI}}}{K_{\mathrm{E,alc}} \times K_{\mathrm{EO,I}}} - \frac{k_3'}{k_2} \qquad (7)$$

Introducing the experimental values for [alc] = 6100 μM, K_{EI} = 45 μM, $K_{EO,I}$ = 2 μM (see Part II), k_3' = 0.0122 $\mu M^{-1} \times sec^{-1}$, k_2 = 3.1 sec^{-1} (Part I) gives $K_{E,alc}$ = 5500 μM, which agrees with the value 4600 μM obtained (see below p. 1842) from the slopes. This confirms that the results presented in Fig. 3 closely conform with noncompetitive conditions. Since $K_{E,I}/K_{E,alc} \times K_{EO,I}$ (0.00406) is only a little larger than k_3'/k_2 (0.00390) the values for [alc] required for strict noncompetition is very sensitive to small errors. If $K_{E,alc}$ is taken 4600 μM (see below), [alc] becomes 1000 μM. This means that very nearly noncompetitive behaviour will be expected with [alc] in the whole region from 1000 μM up to those concentrations where substrate inhibition begins to occur. The [I] required for 50 % inhibition with 6100 μM alcohol is 50 μM according to (6) when $K_{E,alc}$ = 5500 and $K_{E,I}$ = 45 μM. This agrees with the experiments.

The *vice versa* case to caprate, case 3, would be expected to be *iso*butyramide, case 1, since *iso*butyramide gives complexes EI and ERI but not EOI. However, the experimental results, (Fig. 5 a and b), show practically completely *un*competitive behaviour with parallel L-B plots. Substituting $K_{ER,I}$ for $K_{EO,I}$, k_2' for k_2, k_3 for k_3', [ald] for [alc], equation (5) and (7) gives [ald] = 0.15 μM, [I] = 140 μM.

These concentrations, unlike the experiments with caprate, case 3, are very far from the concentrations actually used in the experiments (Fig. 5 a and b). The [ald] used, 370 and 3700 μM, are far above $K_{E.ald}$ (10 μM), and I concentrations are below $K_{E,I}$ (9.3 mM). This is the reason for the different competition pictures.

Rearrangements of the above equations give:

$$\text{Caprate, case 3: } \frac{1}{[I]} = \frac{1}{K_{EO,I}} - \frac{K_{E,alc}}{K_{E,I}} \times \frac{k_3'}{k_2} \qquad (8)$$

where $1/K_{EO,I} = 0.500$ and $K_{E,alc} \times k_3'/K_{E,I} \times k_2 = 0.481$

$$\textit{Iso}\text{butyramide, case 1: } \frac{1}{[I]} = \frac{1}{K_{ER,I}} - \frac{K_{E,ald}}{K_{E,I}} \times \frac{k_3'}{k_2} \qquad (9)$$

where $1/K_{ER,I} = 0.00715$ and $K_{E,ald} \times k_3/K_{E,I} \times k_2' = 0.45 \times 10^{-5}$.
In (8) the first and second terms are nearly equal. Therefore, noncompetition occurs when $K_{EO,I} \approx (K_{E,I} \times k_2)/K_{E,alc} \times k_3')$
In (9) the second term is negligible compared with the first. Therefore
[I] = $K_{ER,I}$ = 140 μM, and [ald] = 0.15 μM
is required to give noncompetitive plots. This experiment cannot be conveniently carried out; [ald] = 0.15 μM is unsufficient to give observable initial reaction rates with excess of DPNH. Therefore possible experiments in case 1 always give more or less uncompetitive plots. In case 3, on the contrary, the constants are of favourable magnitude for giving $\approx$ noncompetitive plots.

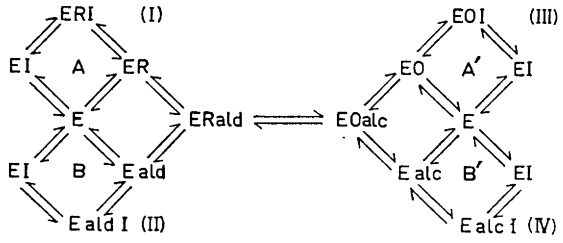
As seen from (7), when it happens that

$$k_3'/k_2 > K_{E,I}/(K_{E,alc} \times K_{EO,I})$$

the substrate concentration should have to be $> \infty$ to fulfil the conditions for noncompetitive inhibition. In such cases mixed noncompetitive-competitive pictures occur. *Ortho*phenanthroline (OP) case 4, is an example of this. In Fig. 1

(Part IV), OP, case 4 approaches competitive behaviour, the [DPN] being 107 μM. The same case done by Vallee *et al.*[6] is also mixed competitive -non-competitive rather than non-competitive as claimed, but the [DPN] being 4000 μM, it is nearer to non-competitive behaviour than with 107 μM DPN. Mixed non- and uncompetitive behaviour results when the substrate concentration is higher than that calculated (eqn. 7) for strict noncompetition.

The possible equilibria between enzyme, substrates and inhibitors are represented by the general mechanism I.



(I)

Only two of the four ternary complexes I—IV are however formed by each of the following inhibitors.

Caprate:	E ald I	and	EOI	(omit I-complexes in A and B′)
IB:	ERI	»	E alc I	(» » » B » A′)
Imidazole:	ERI	»	EOI	(» » » B » B′)
OP:	E ald I	»	E alc I	(» » » A » A′)

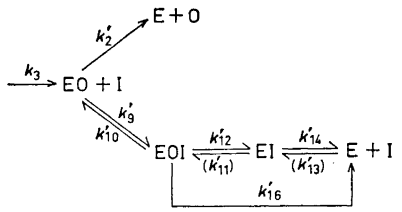
In interpreting the results for each inhibitor the above scheme with the appropriate complexes omitted may be useful. Wherever three compounds are on a straight line those at the ends compete for the middle one. First and second phase effects are also readily apparent. Caprate in the forward reaction gives first phase inhibition through B, second phase inhibition through A′. In the reverse reaction first phase inhibition occurs through A′, but no second phase inhibition occurs, as no ERI is formed.

Table 1. Caprate cases 1 and 2, intercepts. (Corrected to infinite [DPNH] and infinite [ald]).

$[C_{10}]$ μM	e/V		V/e (average) sec^{-1}
	Case 1	Case 2	
0	0.0133	0.0137	74
12.5	0.034		29.4
50	0.086	0.085	11.7
125		0.173	5.8
∞	$1/X$	$1/X$	X

Potassium caprate (C_{10})

Cases 1 and 2, intercepts. The intercepts (Figs. 1 and 2) were first corrected to give the value for $1/V_{max} = 1/k_2'$ at [DPNH] = ∞ and [aldehyde] = ∞, using for case 1 the corresponding slopes in case 2 and the [aldehyde] in case 1, and *vice versa* for case 2. In case 1, the corrections were very small, in case 2, $\sim$ 10 %. The results are given in Table 1. Since [DPNH] is infinite, and ERI is not formed, the inhibition at the intercept must be caused by formation of EOI in the second phase of the reaction:



(A)

If k_9' and k_{10}' are both $\gg k_2'$ and k_{12}', EO and EOI should be in true equilibrium, and the inhibition constant should be $K_{EO,I} = 2$ μM, as determined in equilibrium measurements (see Part II), and confirmed in case 3, *vide infra.*

In case the assumption does not hold, the inhibition constant, calculated from the intercepts, in cases 1 and 2 should be $> K_{EO,I}$, and k_9', k_{10}' and k_{16}' can be calculated.

As seen from scheme A, the free enzyme is regenerated along two pathways, the normal upper one with $V_{max} = k_2' = 74$, the lower, inhibited one with V_{max} = X. The observed V will be the sum of both and as is easily derived, if V_{max}, V/e, and X are the velocities at inhibitor concentrations of zero, I and infinity. Then

$$\frac{V}{e} = \frac{K_I}{[I] + K_I} \times V_{max} + \frac{[I]}{[I] + K_I} \times X \qquad (10)$$

Using equation 10:

$$12.5\ \mu M\ C_{10}\text{: } 29.4 = \frac{K_I}{12.5 + K_I} \times 74 + \frac{12.5}{12.5 + K_I} \times X$$

$$50\ \mu M\ C_{10}\text{ : } 11.7 = \frac{K_I}{50 + K_I} \times 74 + \frac{50}{50 + K_I} \times X$$

$K_I = 7.6$ and $X = 2.23$

Taking the values for 12.5 and 125 μM, C_{10} gives $K_I = 7.8$ and $X = 1.6$. The average is $K_I = 7.7$ μM and $X = 1.9$ sec^{-1} for V_{max} at I = ∞.

In the steady state and at [C_{10}] = 7.7 μM, [EO] = [EOI] + [EI], (E, ER, E ald R and EO alc = 0) and assuming that [EOI] > [EI] which is not unlikely since $K_{E,I}$ (45 μM) is > $K_{EI,O}$ (7 μM) then, as $7.7\ k_9' - k_{10}' = 1.9$ and $k_{10}'/k_9' = 2$ μM = $K_{EO,I}$,
$k_9' = 0.33$ μM^{-1} sec^{-1}, and $k_{10}' = 0.66$ sec^{-1}, $k_{16}' = 1.9$ sec^{-1}.

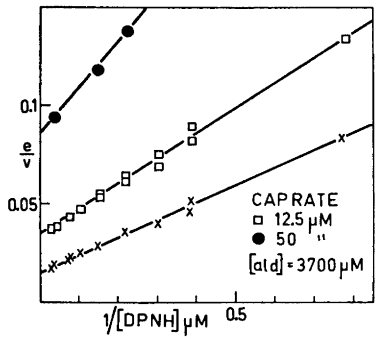


Fig. 1. Case 1. Caprate inhibition. 0.1 μ, 23.5°C and pH 7.
(Points marked × in Figs. 1—16 represent experiments without added inhibitor).

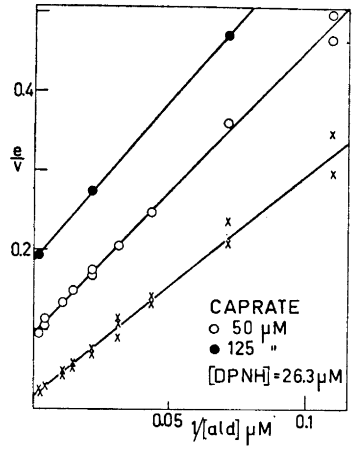


Fig. 2. Case 2. Caprate inhibition. 0.1 μ, 23.5°C and pH 7.

If [EOI] $\gg$ [EI] $k_{12}' = k_{16}'$ ($k_{14}' = \infty$) and as $K_{EI,O} = 7\ \mu M$ (Part II) $= k_{12}'/k_{11}'$, therefore $k_{11}' = 0.27\ sec^{-1}\ \mu M^{-1}$.

k_{11}', the "on" velocity constant for DPN binding with EI, is not far therefore from k_1', the "on" velocity constant for DPN binding with E, 0.5 sec^{-1} μM^{-1}. Since $K_{E,O} = 160\ \mu M$ and $K_{EI,O}$ is 7 μM this means that the introduction of the fatty acid into the enzyme molecule only causes a small, if any decrease in "on" velocity, but a drastic reduction of the "off" velocity, from 74 to 1.9 sec^{-1}. Similar examples of what may be a general rule will be given below.

Case 1, slopes (Fig. 1). In addition to the second phase effect on the intercepts already commented upon, there is an increase in slope with increasing concentrations of C_{10}.

The difference in intercepts above was shown to be a second phase effect. The slopes therefore may indicate competition between DPNH and C_{10} in the first phase. This is very likely because DPNH and C_{10} do not form ternary complexes with the enzyme, which is most easily explained by assuming competition for the same binding site. The constant aldehyde concentration is very

Table 2. Caprate, case 1, slopes.

$[C_{10}]$ μM	Slopes, $\mu M \times sec$	K_I
0	0.088	—
12.5	0.126	29
50	0.244	31
		Average 30 μM

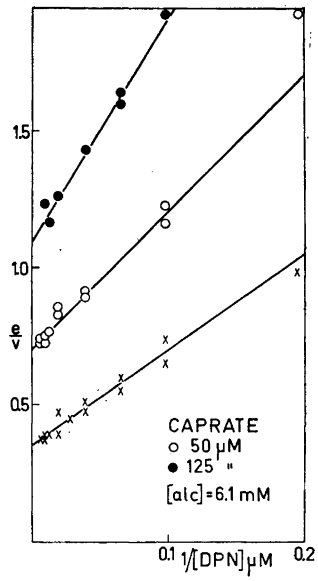


Fig. 3. Case 3. Caprate inhibition. 0.1 μ, 23.5°C and pH 7.

high compared with $K_{E,ald}$ (see below) and there will be very little free enzyme. The enzyme at low concentrations of DPNH starts a new cycle by forming the binary Eald complex which at low [DPNH] could be in equilibrium with C_{10} as E ald I. K_I would then reflect E ald + I $\rightleftharpoons$ E ald I and be $K_{Eald,I}$. The value 30 μM (Table 2) is close to $K_{E:I}$ (45 μM).

Case 2, slopes. The slopes show nearly uncompetitive inhibition caused by the formation of EOI in the second phase. The small differences in the slopes are due to some EI being formed since [DPNH] is not infinite, and a small part of E will be free to be inhibited as EI.

Case 3, intercepts. At the intercept (Fig. 3) 50 μM caprate causes just 50 % inhibition that occurs in the first phase, since only EOI can be formed. The situation in the steady state may be described as follows. When [DPN] goes to infinity, all free E will immediately form EO. The alcohol concentration is high (6100 μM) but not infinite. When the overall reaction velocity is reduced to half by 50 μM C_{10}, 50 % of the total enzyme appears as inactive EOI. Using the rate constants from Part I, $k_3' = 0.0122\ \mu M^{-1} \times sec^{-1}$ and $k_2 = 3.12\ sec^{-1}$, $0.0122 \times 6100 \times EO = 3.12 \times ER$, (because EO + alc $\rightarrow$ and ER $\rightarrow$ E + R are consecutive) and as [ER] + [EO] = 0.5 [E_t] = [EOI], therefore [EO] = 0.02 [E_t], [ER] = 0.48 [E_t] and $K_{EO,I} = 0.02 \times 50\ \mu M/0.50 = 2\ \mu M$, in agreement with the equilibrium value reported in Part II.

Case 4, intercepts. Figure 4 a and particularly 4 b show strict competition of C_{10} with alcohol, with intercept at the same point on the ordinate. This is to be expected since ERI is not formed, and no interference in the second phase is therefore to be expected, in agreement with the earlier results.

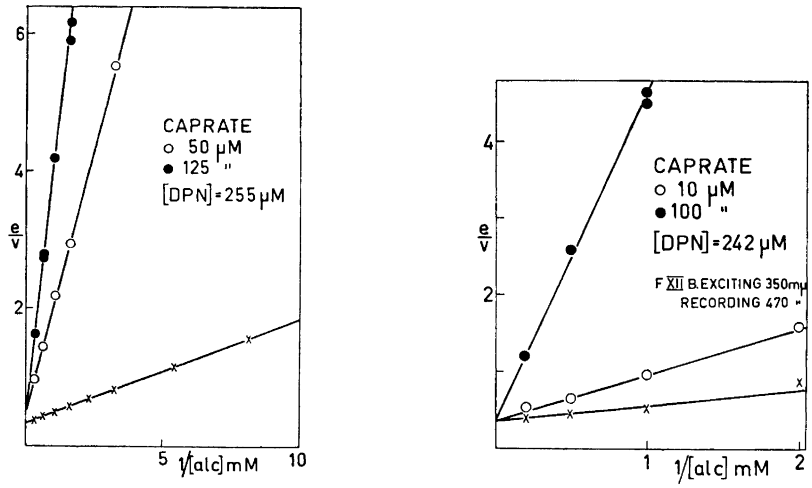


Fig. 4 a and b. Case 4. Caprate inhibition. 0.1 μ, 23.5°C and pH 7. In 4b the experiment was performed on the spectrophotofluorometer.

Case 3, slopes. Since C_{10} and alcohol are competitive, when [DPN] → 0, with [alc] = 6100 μM, and $[C_{10}]$ = 104 μM (Table 3), the following equilibrium will prevail

E alc ⇌ E ⇌ EI,

where [E alc] + [E] = [EI], [EI] = 0.5 $[E_t]$ and [E] = X $[E_t]$.

Therefore [E alc] = (0.5—X) × $[E_t]$, and taking $K_{E,I}$ = 45 μM, gives X = 0.216 and $K_{E.alc}$ = 4600 μM. This is not far from the value $K_{E,alc}$ = 6100 μM, obtained from the Michaelis constants (see part I).

Case 4, slopes. The contrast between this strict competition and the uncompetitive behaviour of caprate and aldehyde is marked and emphasizes that aldehyde and alcohol must have different binding sites. When [alc] → 0 the following equilibria will prevail

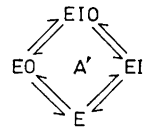


Table 3. Caprate, case 3, slopes.

$[C_{10}]$ μM	Slopes, sec × μM	K_I
0	3.5	
50	5.1	113
125	8.1	95
		Average 104 μM

Table 4. Caprate, case 4, slopes.

Expt.	$[C_{10}]$ μM	Slopes, μM × sec	K_I
R XXVI (fluorometer)	0	150	
	50	1 544	4.8
	125	3 560	5.2
F XII B (spectrophotofluorometer)	0	200	
	10	600	5.0
	100	4 320	4.9
			Average 5.0 μM

When [I] = 5.0 μM (Table 4); [EI] + [EIO] = [E] + [EO]. With $K_{E,O}$ = 160 μM, [DPN] = 255 μM and $K_{E,I}$ = 45 μM: $K_{EO,I}$ = 3.2 μM, in fair agreement with the values of 2 μM obtained from both equilibrium measurements (Part II) and from the intercepts in case 3.

*Iso*butyramide (IB)

*Case 1, intercepts. Iso*butyramide causes an uncompetitive type of inhibition which is dependent on the aldehyde concentration (IB and DPNH can both go on to the enzyme). As can be seen from Fig. 5 a and 5 b, it is much larger with 370 μM than with 3700 μM aldehyde. Table 5 gives the values obtained by extrapolation of the intercepts to infinite [aldehyde], using the slopes in case 2.

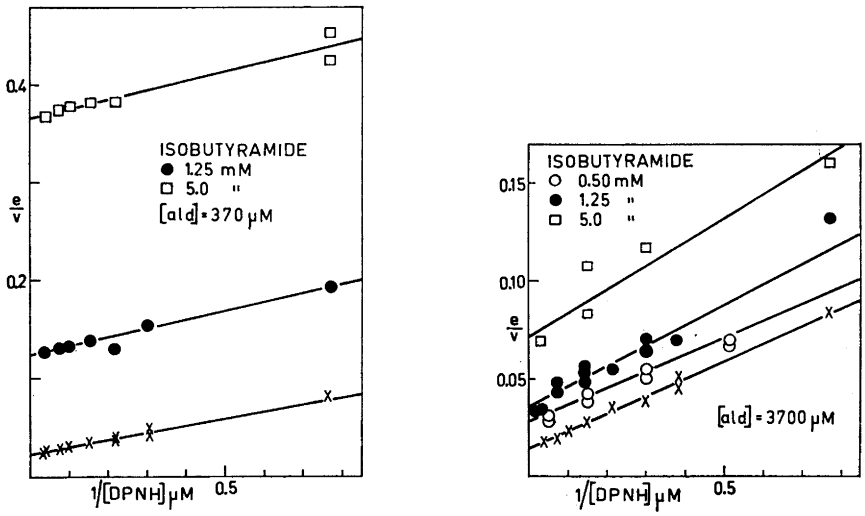


Fig. 5 a and b. Case 1. *Iso*butyramide inhibition. 0.1 μ, 23.5°C and pH 7.

Table 5. Isobutyramide, case 1 intercepts (Uncorrected and corrected to infinite [DPNH] and [ald])

[IB.] mM	e/V			
	A (370 μM [ald])		B (3 700 μM [ald])	
	Uncorr.	Corr.	Uncorr.	Corr.
0	0.022	0.015	0.0145	0.0132
1.25	0.124	0.025	0.036	0.029
5	0.366	0.030	0.071	0.044

The values for e/V_{max} at [ald] = ∞ in the presence of IB, do not quite go down to the values without IB, but the corrections are large and the results therefore uncertain. In case the difference between V_{max} with and without IB are real, they would indicate formation of EOI ($K_{EO,I} \approx 5$ mM) at high concentrations of IB. However, since the Lineweaver-Burk plots in case 2 seem to cross on the ordinate, and as the equilibrium measurements gave no indication of EOI formation [1], the earlier conclusion seems valid, that EOI is not formed, or perhaps only at such high concentrations that EOI plays no essential role in the kinetics.

The intercepts obtained in case 1a, (Fig. 5) can be used for checking either $K_{ER,I}$ or k_3 as follows.

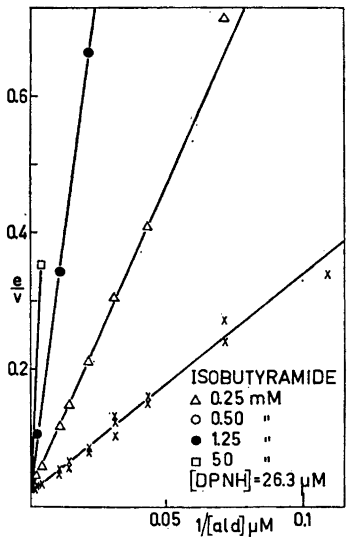
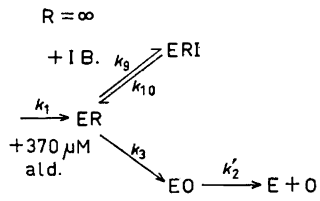


Fig. 6. Case 2. *Iso*butyramide inhibition. 0.1 μ, 23.5°C and pH 7.



(B)

From the intercepts K_I = 300 μM, $K_{ER,I}$ (Part II) = 140 μM, and thus ER/ERI = 0.466. At half inhibition [ERI] = [ER] + [EO] = 0.5 [E_t] and therefore [ER] = 0.233 [E_t], and [EO] = 0.267 [E_t]. As k'_2 = 74 sec^{-1},
370 × 0.233 × k_3 = 74 × 0.267, and therefore
k_3 = 0.23 $\mu M^{-1} \times sec^{-1}$
in fair agreement with the value 0.31 obtained from the kinetic measurements without inhibitors (Part I).

Case 1, slopes. The inhibition is nearly uncompetitive for reasons discussed above p. 1836, or in other worlds $K_{E,I} >> K_{ER,I}$.

Case 2, intercepts. Fig. 6 shows competitive inhibition as found earlier [1], the intercepts meeting at the same point on the ordinate and no EOI being formed.

Case 2, slopes. Since the concentration of DPNH is high compared with $K_{E,R}$, and $K_{E,I}$ is very high, the inhibition constant K_I = 143 mM (Table 6) in this case is $K_{ER,I}$, in excellent agreement with the value (140 mM) obtained from the equilibrium determinations (Part II).

Cases 3 and 4, intercepts (Figs. 7 and 8). Inhibition occurs in the second phase due to the presence of ERI. The data in Table 7 when treated as for caprate, cases 1 and 2 (above), gave X = 0.077 sec^{-1} and K_I = 200 μM. Therefore, $k_{12} = k_{16}$ = 0.077 sec^{-1} (scheme C), since in this case it can be taken for certain that $k_{14} >> k_{12}$: $K_{E,I}$ (9300 μM, Part II) is 2 million times greater than $K_{EI,R}$ (0.005 μM). In Part II, a value for k_{11} = 14 sec^{-1} μM^{-1} was obtained from direct reaction cycles in very dilute solutions, thus $K_{EI,R}$ = 0.077/14 = 5.5 × 10^{-3} μM, in excellent agreement with the equilibrium titrations in Part II.

Table 6. Isobutyramide, case 2, slopes.

[IB] mM	Slope, μM × sec	$K_I = K_{EE,I}$
0	2.8	
0.25	7.8	138
0.50	12.3	145
1.25	26	148
		Average 143 μM

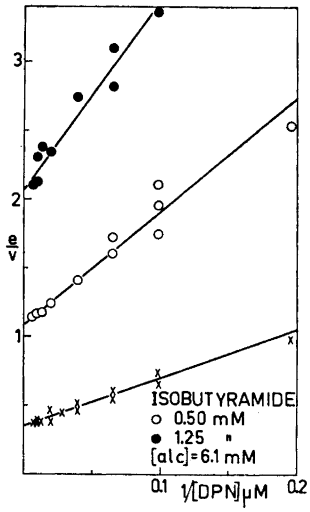


Fig. 7. Case 3. *Iso*butyramide inhibition. 0.1 μ, 23.5°C and pH 7.

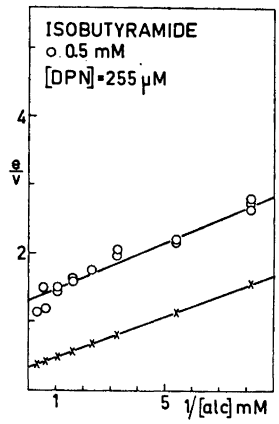
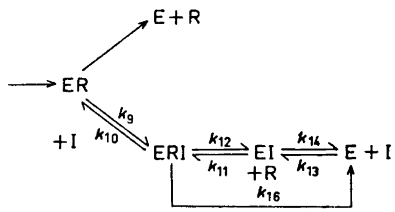


Fig. 8. Case 4. *Iso*butyramide inhibition. 0.1 μ, 23.5°C and pH 7.



(C)

Taking $K_{ER,I\,eq} = k_{10}/k_9 = 140$ μM, and [I] = 200 μM, where [ER] = [ERI]; $0.077 = 200\ k_9 - k_{10} = k_{16}$

$k_9 = 1.3$ sec$^{-1} \times$ mM^{-1} and $k_{10} = 0.18$ sec^{-1}.

Case 3, slopes. No EOI is formed and Fig. 7 accordingly shows first phase competition between DPN and IB. Since I and alcohol do not compete with one another, it may be that a ternary complex E alc I can be formed. Consider-

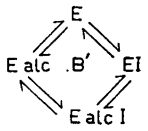
*Table 7. Iso*butyramide, cases 3 and 4, intercepts (Corrected to infinite [DPN] and [alc]).

[IB] mM	e/V			V/e
	Case 3	Case 4	Average	
0	0.330	0.329	0.33	3.0
0.5	1.045	1.145	1.10	0.91
1.25	2.01	2.14	2.08	0.48
∞				X

Table 8. Isobutyramide, case 3, slopes.

[IB] mM	Slopes, μM × sec	K_I
0	3.5	
0.5	8.3	365 μM
1.25	15.8	355 "

ing that $K_{E,I}$ is 9.3 mM, very little inhibition would occur at [I] = 0.5 and 1.25 mM, if only EI were formed. In the equilibrium

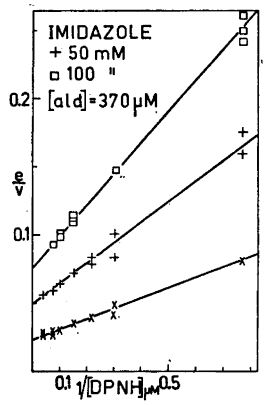


when [DPN] → 0, and when [I] = 360 μM (Table 8), [E] + [E alc] = [EI] + [E alc I]. From this equation, and from the values for $K_{E,I}$ = 9300 and $K_{E,alc}$ = 4600 μM, [Ealc I] can be calculated, giving $K_{EI,alc}$ = 103 μM and $K_{E\,alc,I}$ = 209 μM. Thus there is high stabilisation operating between alcohol and isobutyramide; the stabilisation factor being 4600/103 = 45. This is different from the complex E alc caprate (see p. 1841) where the stabilization was very low.

Case 4, slopes. No appreciable change in slope (uncompetitive and no inhibition in the first phase) occurs on addition of *iso*butyramide (Fig. 8) because EOI is not formed, and the concentrations of I used were too low compared with $K_{E,I}$ to cause the formation of more than a small fraction of EI.

Imidazole, pH 7

Cases 1 and 2, intercepts. Case 1 was carried out with two different concentrations of aldehyde, 370 μM (Fig. 9 a) and 3700 μM (Fig. 9 b).



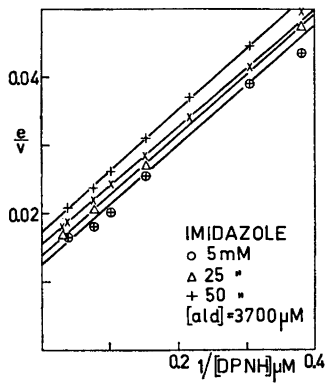


Fig. 9 a and b. Case 1. Imidazole. 0.1 μ, 23.5°C and pH 7.

Table 9. Imidazole, cases 1 and 2, intercepts (Corrected to infinite [DPNH] and [ald]).

[Im] mM	e/V (average)	V/e
0	0.0135	74
5	0.0096	104
10	0.0097	103
20	0.0086	116
25	0.0083	120

Fig. 9 b shows an uncompetitive picture with smaller concentrations of imidazole causing slight stimulation, higher ones some inhibition. However, if the intercepts were corrected to infinite [ald], then the results shown in Table 9 for the averages of cases 1 and 2 were obtained. There is thus a slight increase in reaction velocity in the last phase, but as seen below the influence of imidazole is slight as EO here decomposes almost as fast as EOI. At pH 9 the presence of EOI will be seen to be much more noticeable, the stimulation observed being much greater. Using the values for 5 and 20 mM imidazole in eqn. 10, $X = 122$ and $K_{EO,I\,kin} = 3.1$ mM; 5 mM and 25 mM gave $X = 126$ and $K_{EO,I\,kin} = 3.8$ mM.

The value for 10 mM did not fit with these values. It should be remembered, however, that the errors in these experiments are such that better agreement cannot always be expected. The values for $K_{EO,I}$ fit with the value $K_{EO,I}$ (uncorr.) at pH 7 of 3.3 mM obtained by equilibrium measurements in Part II.

The destabilization factor operating between I and DPN at pH 7 is from the equilibrium data in Part II: $K_{EI,O}/K_{E,O} = 440/160 = 2.75$.
Taking V_{max} for the decomposition

$$EIO \underset{k_{11}'}{\overset{k_{12}'}{\rightleftharpoons}} EI \underset{k_{13}'}{\overset{k_{14}'}{\rightleftharpoons}} E = 124 \text{ sec}^{-1} \text{ (see scheme A)}$$

$$\text{then } \frac{1}{k_{12}'} + \frac{1}{k_{14}'} = \frac{1}{124}$$

Since $K_{EI,O} = 440$ μM and $K_{E,I\,corr} = 550$ μM (Part II) are of the same order of magnitude, it is reasonable to assume that k_{12}' and k_{14}' should in this case be of similar order of magnitude and both contribute to the time needed for the liberation of E from EOI. If the "on" velocity constant k_{11}' ($EI + O \xrightarrow{k_{11}'}$) is assumed to be the same as k_1' ($E + O \xrightarrow{k_1'}$) $= 0.53$ sec^{-1} $\times$ μM^{-1} (which is probably not far from true, *cf.* caprate, cases 1 and 2, and *iso*butyramide cases 3 and 4) then $k_{12}' = 0.53 \times 440 = 234$ sec^{-1}. Since V_{max} for [I] $= \infty$ is 124 sec^{-1}, $1/234 + 1/k_{14}' = 1/124$ and $k_{14}' = 257$ sec^{-1}, $K_{E,I} = 550\,\mu M = k_{14}'/k_{13}'$, therefore $k_{13}' = 0.47$ μM^{-1} $\times$ sec^{-1}.

These values are only to be taken as a general confirmation of the mechanism. However, if we assume k_{14}' and k_{13}' both to be very large, $k_{12}' = 124$ sec^{-1} and $k_{11}' = 0.28$ μM^{-1} $\times$ sec^{-1}, which is not very far from the values

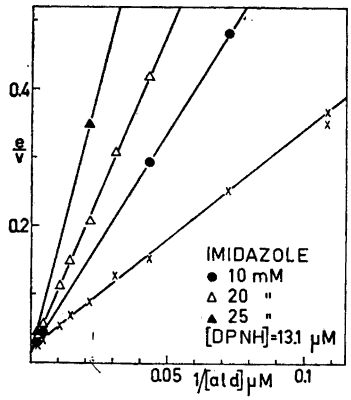


Fig. 10. Case 2. Imidazole 0.1. μ, 23.5°C and pH 7.

of $k_{11}' = 0.53$ under the first assumption of $k_{11}' = k_1'$. Whereas k_{11}' is thus reasonably well defined the absolute values of k_{13}' and k_{14}' are in fact unknown. They are only bound by the relation $k_{14}'/k_{13}' = 550$ μM.

Case 1a, slopes: With 370 μM aldehyde (Fig. 9a), nearly noncompetitive inhibition happens to occur at this [ald]. Compare the same case at pH 9 (Fig. 13 a and b).

As case 2 shows, imidazole competes strictly with aldehyde. At very low [DPNH] most of the enzyme exists in the equilibrium E ald $\rightleftharpoons$ E $\rightleftharpoons$ EI, where [E] is very small. The slopes give $K_I = 44.5$ mM imidazole. At 370 μM aldehyde and 44.5 mM imidazole therefore [E ald] = [EI], and $K_{E,ald} = 10.2$ μM ($K_{E,I\ uncorr.}$ being 1.23 mM). This value agrees with $K_{E,ald} = 10.0$ μM, obtained from the Michaelis constants (see Part I).

Case 2, slopes. These (Fig. 10) give $K_{ER,I}$, since the enzyme is at low [ald] essentially in the equilibrium ER + I $\rightleftharpoons$ ERI. The value of 8.9 mM (Table 10) agrees rather well with the value obtained in Part II ($K_{ER,I\ uncorr.} = 8$ mM).

Cases 3 and 4, intercepts. As seen from Figures 11 and 12 the plots without or with different concentrations of imidazole cross one another in an interesting way. At high concentrations of both DPN and alcohol, imidazole causes a considerable increase in reaction velocity, which at the intercepts, when corrected to infinite concentration of both DPN and alcohol, is 4—5 fold for 10 mM imidazole, 7—10 fold for 20 mM.

Table 10. Imidazole, case 2, slopes.

[Im] mM	Slope, μM × sec	$K_I = K_{ER,I}$
0	2.99	—
10	5.98	10.0
20	8.70	10.4
25	16.0	6.3
		Average 8.9 mM

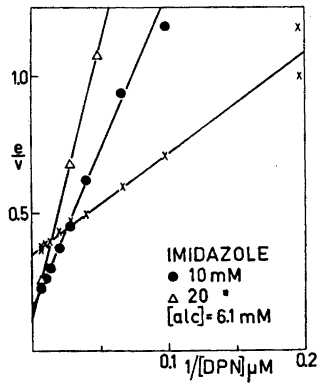


Fig. 11. Case 3. Imidazole. 0.1 μ, 23.5°C and pH 7.

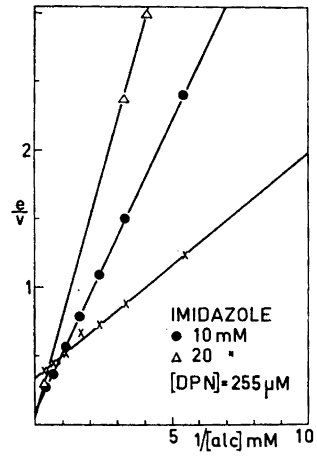


Fig. 12. Case 4. Imidazole. 0.1 μ, 23.5°C and pH 7.

This is explained by the fact that under these conditions without imidazole practically all of the enzyme is in the form of ER, and the dissociation velocity of

$ER \xrightarrow{k_2} E + R$, is rate limiting.

In the presence of imidazole, ERI is formed, which is less stable than ER. At pH 7, the destabilization factor is (*cf.* Part II) $K_{EI,R}/K_{E,R} = 2.01/0.31 = 6.5$, in general agreement with the extent of rate increase observed. As already discussed for caprate inhibition (cases 1 and 2) and *iso*butyramide inhibition (cases 3 and 4), the changes in dissociation constants caused by ternary complex formation are caused essentially by changes in the dissociation rate constants; the association rate constants seem rather independent of the presence or absence of another ligand.

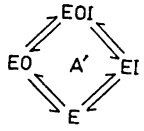
The intercept data in this case do not allow accurate calculations because the extrapolations to infinite concentrations of DPN and alcohol become quite large at pH 7, where alcohol cannot be added in sufficient excess because of the inhibition effects described in Part II. Conditions are more favorable at pH 9, where such calculations are made (see below).

Case 3, slopes. It is noteworthy that imidazole and DPN compete, although as seen later from the stimulation shown at pH 9 (cases 1 and 2) and as shown in Part II, EOI is also formed. This competition is probably due to a charge effect, as discussed below, p. 1859.

In Fig. 11, $K_I = 4.7$ mM for both 10 and 20 mM imidazole. As in the case of aldehyde, assuming a complex E-alc-imidazole to be formed led to unreasonable results. Assuming strict competition, $K_I = 4.7$, $K_{E,I} = 1.23$ and [alc] = 6.1 mM gives $K_{E,alc} = 2.2$ mM.

This value is considerably lower than $K_{E,alc}$ obtained from the caprate inhibition (4.6 mM). The latter value is considered more likely to be true, since it is closer to the value $K_{E,alc} = 6$ mM obtained from the Michaelis constant in Part I.

Case 4, slopes (Fig. 12). There is strict competition with alcohol along with a second phase effect. At very low alcohol concentration, the following equilibria are assumed to prevail:



The concentration of imidazole expected to give half inhibition, where [E] + [EO] = [EI] + [EIO] was calculated from the data given in Parts I and II; $K_{E,O} = 160$ mM, $K_{E,I\ uncorr.} = 1.23$ mM, $K_{EO,I\ uncorr.} = 3.3$ mM, and $K_{I,calc}$ was found to be 2 mM. However, the experimental K_I is considerably higher, being 6.2 for 10 mM imidazole, 5.8 for 20 mM. The average $K_I = 6$ mM, is thus three times higher than the value calculated from the equilibrium measurements.

Imidazole pH 9

Cases 1 and 2, intercepts. The intercepts in Figs. 13 and 14 were corrected to infinite concentrations of DPNH and aldehyde (Table 11). Calculations using eqn. 1 gave $K_{EO,I} = K_I = 30.8$ mM and $X = 44.4$ sec^{-1}.

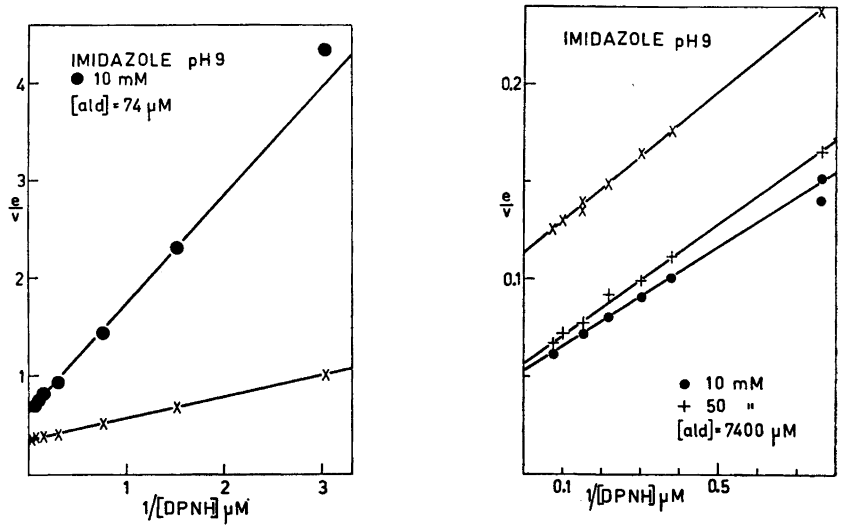


Fig. 13 a and b. Case 1. Imidazole. 0.1 μ, 23.5°C and pH 9.

Table 11. Imidazole, pH 9, cases 1 and 2, intercepts (corrected to infinite [DPNH] and [ald]).

[Im] mM	V/e, average
0	8.1
2	10.3
10	17.0
50	30.6
∞	X

A 5-fold stimulation of the reaction velocity is thus produced, due to the formation of EOI, which decomposes at a faster rate, 44 sec^{-1}, than EO, 8.1 sec^{-1}.

$K_{EO,I}$ (Part II) = 24.2 mM = k_{10}'/k_9' and $k_9' \times 30.8 - k_{10}' = 44.4$. Therefore $k_9' = 7\ mM^{-1} \times sec^{-1}$ and $k_{10}' = 168\ sec^{-1}$.

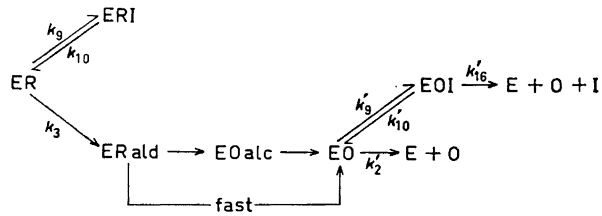
The relation between k_{12}' and k_{14}' in this case cannot be predicted since $K_{E,I}$ (670 μM) and $K_{EI,O}$ (440 μM) are rather equal. Probably both k_{12}' and k_{14}' contribute to the time factor e/V in the equation

$e/V = 1/k_{12}' + 1/k_{14}' = 1/k_{16}' = 1/44.4$

which obtains for infinite [DPNH], [aldehyde] and [imidazole].

Fig. 13 a, with 74 μM aldehyde, is interpreted as a stimulating second phase effect, combined with a non-competitive first phase effect.

Second phase effect: The reciprocals of the intercepts were 2.8 sec^{-1} without and 1.4 sec^{-1} with 10 mM imidazole. This can be used to calculate $K_{ER,I}$. Since R = ∞ at the intercept, the reaction may be considered to be:



(D)

The decomposition of EO needs to be considered only as the summarized rate constant = 1.4 sec^{-1}. As $k_3 = 0.062\ \mu M^{-1}\ sec^{-1}$ (Part I), and the enzyme exists only in the three forms ER, EO and ERI, where at half inhibition, caused by 10 mM imidazole, [ERI] = [ER] + [EO]. Therefore $0.062 \times 74 \times [ER] = 1.4 \times [EO]$, from which $K_{ER,I}$ = 2.4 mM, in reasonable agreement with the equilibrium value 3.0 mM (See Part II, Table 15).

Case 1, slopes. The slopes as at pH 7 are nearly independent of [Im] when [ald] is high (Fig. 13 b).

First phase effect: When [ald] is lower as in Fig. 13 a where [ald] is 74 μM, the slopes differ due to competition between imidazole and aldehyde. Without imidazole the slope is 0.22 μM × sec, while with 10 mM imidazole it is 1.10 μM × sec, giving K_I = 2.5 mM.

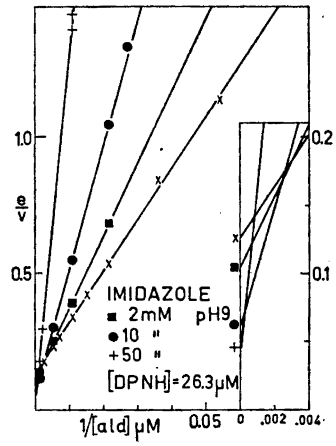


Fig. 14. Case 2. Imidazole. 0.1 μ, 23.5°C and pH 9. The intercepts enlarged and corrected to infinite [DPNH] are also shown.

When [DPNH] goes to zero with [I] = 2.5 mM and [ald] = 74 μM, [EI] = [E] + [Eald] and $K_{E,I\ uncorr.} = 0.68 = 2.5[E]/[EI]$.
Therefore [EI] = 3.7 [E], [E ald] = 2.7 [E], and $K_{E,ald} = 27$ μM.*

Case 2, slopes. The slopes in Fig. 14 indicate competition between imidazole and aldehyde. K_I (Table 12) would be expected to correspond to $K_{ER,I}$ in this case, which was found to be 3 mM in the equilibrium experiments.

The drift with [Im] and the not too good agreement with the equilibrium value may depend upon oversimplified assumptions.

Cases 3 and 4, intercepts (Fig. 15 and 16). The intercepts (Table 13) were corrected to infinite [DPN] and [alc], using eqn. 1.
2 and 10 mM imidazole: $K_I = 10.4$ mM, $X = 22$ sec^{-1}
10 and 50 mM imidazole: $K_I = 10.6$ mM $= 22.2$ sec^{-1}
Thus at 10.5 mM imidazole, [ER] = [ERI] (see scheme C) in the "second phase" of the reaction.

$K_{ER,I}$ (Part II) = 3 mM $= k_{10}/k_9$ and $k_9 \times 10.5 - k_{10} = 22.1$. Therefore $k_9 = 3$ mM$^{-1} \times$ sec^{-1} (cf. 7 mM$^{-1} \times$ sec^{-1} at pH 7 and $k_{10} = 9$ sec^{-1}). The "destabilization factor" for ERI is $K_{ER,I}/K_{E,I} = 3/0.67 = 4.5$.

Table 12. Imidazole, pH 9, case 2, slopes.

[Im] mM	Slope, μM × sec	K_I
0	18.8	
2	26.4	5 mM
10	45.2	7
50	126	9

* Recent determinations of Φ-values and rate constants at pH 9 (H. T. and L.-G. Falksveden) gave $K_{m,ald}$ (DPNH → O) = $K_{E,ald} = k_2/k_3 = 5.0/0.096 = 52$ μM. The value found from the competition between aldehyde and imidazole is again some two times lower.

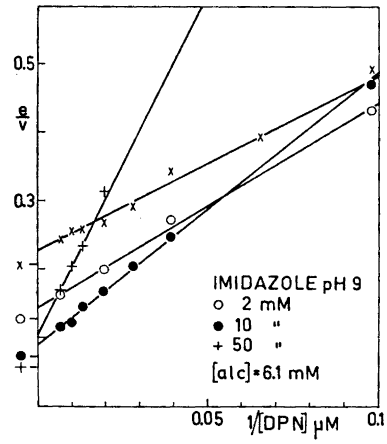


Fig. 15. Case 3. Imidazole. 0.1 μ, 23.5°C and pH 9. The position the intercept has when corrected to infinite [alcohol] is shown.

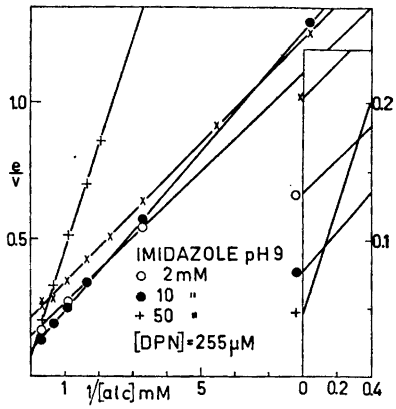


Fig. 16. Case 4. Imidazole. 0.1 μ, 23.5°C and pH 9. The intercepts enlarged and corrected to infinite [DPN] are also shown.

If it is assumed that the "on" velocity constants for R being attached to E (k_1) and to EI (k_{11}) are equal, the ratio between the dissociation constants should depend entirely on a corresponding difference in the "off" velocities.

In this case k_{12} should be $4.5 \times k_2 = 4.5 \times 4.9 = 22$ sec^{-1}. The agreement is surprisingly good; assuming that k_{14} is $\gg k_{12}$, which is reasonable since $K_{E,I} = 680$ μM and $K_{EI,R} = 2.91$ μM, $k_{12} = k_{16} = 22$ sec^{-1}. Again it appears probable that the changes in dissociation constants occurring on ternary complex formation are predominantly caused by changes in the "off" velocity constants of the ligands.

Case 3, slopes. As seen from Fig. 15, the slopes indicate competition by charge effect between DPN and imidazole, as was the case at pH 7. Presuming that when [DPN] approaches zero the equilibrium EI $\rightleftharpoons$ E $\rightleftharpoons$ E alc is maintained, then using $K_{E,I\ uncorr.} = 0.68$ mM, $K_I = 17.8$ mM (Table 14) and [alc] = 6.1 mM, $K_{E,alc}$ is found to be 0.23 mM. This value is again lower than that expected from K_m at [DPN] $\rightarrow 0$; $k_2'/k_3' = 1$ mM.

Case 4, slopes. The slopes in Fig. 16 again show competition. They differ markedly from the slope without imidazole (128 μM $\times$ sec) only in the presence

Table 13. Imidazole, pH 9, cases 3 and 4 intercepts (uncorrected and corrected to infinite [alc] and [DPN]).

	e/V				Average	
[Im] mM	Case 3		Case 4		*e/V*	*V/e*
	Uncorr.	Corr.	Uncorr.	Corr.		
0	0.225	0.209	0.210	0.200	0.204	4.9
2	0.144	0.128	0.145	0.134	0.131	7.6
10	0.090	0.0736	0.090	0.0766	0.075	13.3
50	0.104	0.057	0.080	0.046	0.052	19.2
∞						*X*

Table 14. Imidazole, pH 9, case 3, slopes.

[Im] mM	Slopes, μM × sec	K_I
0	2.58	—
(2	3.0)	—
10	4.0	18.2
50	10	17.4
		Average 17.8 mM

of 50 mM imidazole (384 μM × sec), from which K_I = 25 mM. From the equilibrium measurements in Part II it was known that EOI is formed. Calculating the [Im] that would be needed for fulfilling the half inhibition condition when [alc] → zero, [E] + [EO] = [EI] + [EOI] (see pH 7), using the equilibrium constants from Part II gives $[Im]_{50\ \%\ inhibition}$ = 9.8 mM. This value is again ~ 3 times lower than the experimentally found 25 mM, just as was the case at pH 7. On the other hand K_I = 25 mM happens to agree with $K_{EO,I}$ = 24.6 mM, found in Part II.

Ethanol

In Part II the formation of a complex ER alc at high concentrations of alcohol, with $K_{ER,alc}$ = 40 mM, $K_{E,alc}$* = 100 mM, and $K_{E,alc,R}$ = 0.12 μM was observed. Kinetic experiments presented in Fig. 17 indicated that at infinite alcohol concentration e/V is = 0.75 instead of the value 0.3 obtained by straight line extrapolation from values at [alc] < 10 mM. Mutual stabilization thus occurs with a factor of 2.5, ($K_{ER}/K_{E,alc,R}$ = 0.31/0.12 = $K_{E,alc}$*/$K_{ER,alc}$ = 2.5), which corresponds to the decrease in reaction velocity at infinite alcohol concentration, 0.75/0.3 = 2.5. As already found for the ternary complexes with caprate, *iso*butyramide and imidazole, the introduction of one ligand does not interfere with the "on" velocity constant of the other, but only with the "off" velocity constant:

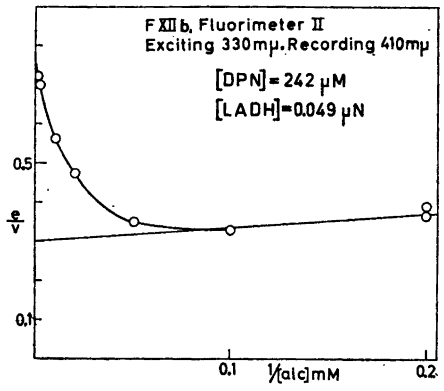


Fig. 17. Kinetics in the presence of relatively high concentrations of alcohol. The spectrophotofluorometer was used.

$$\text{E alc}^* + \text{R} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{E alc R}$$

$1.25/k_{on} = 0.12$; $k_{on} = 10.4\ \mu M^{-1} \times sec^{-1}$ in agreement with $k_1 = 11.2\ \mu M^{-1} \times sec^{-1}$ (Part II).

Half inhibition, $e/V = 0.525$, occurs at 75 mM alcohol. This kinetic value is, as expected, somewhat higher than the equilibrium value ($K_{ER,alc} = 40$ mM) and the differences could be used for calculating the "on" and "off" velocity constants for alcohol in equilibrium with ER, as was done above for example with *iso*butyramide (scheme B, I = alcohol). This would give $k_9 = 0.036\ mM^{-1} \times sec^{-1}$, $k_{10} = 1.4\ sec^{-1}$. However, the value 40 mM is uncertain, so that no great attention should be paid to the absolute magnitude, but it should be noticed that k_9 is very low, perhaps reflecting the low probability of a successful collision between ER and alcohol to form the abortive ER alc complex.

DISCUSSION

"On" and "off" velocities in binary and ternary complexes

$K_{E,ald}$ and $K_{E,alc}$ determined from the imidazole case 1, and caprate, case 3, experiments, agreed with the values determined in Part 1 from $K_{M,ald}$ and $K_{M,alc}$ when the coenzyme concentration approached zero. This further substantiates the existance of the complexes E ald and E alc, and the general two substrate mechanism A (Part I).

As discussed in Part I the "on" velocity constants for DPNH and DPN are nearly independent of whether DPNH is attached to E or to E,ald (k_1, resp. k_7), or DPN to E or to E alc (k_1', resp. k_7').

There is convincing evidence to believe that the maximum velocity in the forward reaction is k_2' ($EO \xrightarrow{k_2'} E + O$) and in the backward reaction, k_2 ($ER \xrightarrow{k_2} E + R$). The values found for k_2' were at pH 7, 74 sec^{-1}; at pH 9, 8.2 sec^{-1}; for k_2 at pH 7, 3.1 sec^{-1}; at pH 9, 4.9 sec^{-1}.

The dissociation constants $K_{E,R}$ and $K_{E,O}$, determined by equilibrium experiments in the absence of substrate (see Part II), together with the "off" velocity constants from the kinetic experiments allowed the calculation of the "on" velocity constants k_1 ($E + R \xrightarrow{k_1} ER$) and k_1' ($E + O \xrightarrow{k_1'} EO$).

The kinetic "on" velocity constants, as discussed in Part I, reflect k_7 ($E\ ald + R \xrightarrow{k_7} E\ ald\ R$) and k_7' ($E\ alc + O \xrightarrow{k_7'} E\ alc\ O$). The relations found between these constants were:

	k_7/k_1	k_7'/k_1'
pH 7	0.90	0.88
pH 9	0.76	0.75

The conclusion was that substrates on their binding sites at the enzyme only slightly affect the association velocity of their coenzyme partner. Nothing could be said, however, from these experiments as to how the presence of substrates in ternary complexes would affect the "off" velocity of the coenzyme, because these ternary complexes are rapidly transformed to other products and their dissociation constants therefore still unknown. This is not the case with ternary complexes of enzyme, coenzyme and inhibitors, and the experiments reported herein show that whereas the "on" velocity for the coenzyme is scarcely changed by inhibitors at the substrate site, the "off" velocity for the coenzyme is greatly affected, being decreased with caprate and *iso*butyramide, but increased with imidazole. For *caprate* at pH 7, k_{11}' (EI + O $\xrightarrow{k_{11}'}$ EIO) is at least = 0.54 × k_1' ,and k_{11}' may easily be $k_1' \cdot k_{16}'$ (EIO $\xrightarrow{k_{16}'}$ E + I + O), on the contrary, is only = 0.026 × k_2', so that the dissociation velocity of DPN is reduced fortyfold by caprate attached to the binding site of alcohol.

In the case of iso*butyramide* the constant k_{11} (EI + R $\xrightarrow{k_{11}}$ EIR) could be determined directly from reaction cycles at 10^{-8} M concentrations along with $K_{EI,R}$. k_{11} was found to be 14 μM^{-1} sec^{-1}, which is $\approx k_1$, whereas k_{16} was 0.025 × k_2, a fortyfold decrease just as in the case of caprate.

Imidazole forms ternary complexes corresponding to both ERI and EOI. At pH 7 the formation of EOI in the second phase of the forward reaction causes a slight increase in the maximal reaction velocity, from 74 to 124 sec^{-1}. This increase (the "destabilization factor"), 124/74 = 1.7, agrees with the increase in $K_{E,O}$ when the pH is lowered from 7 to approach the acid asymptote (288/160 = 1.8). Both cases may reflect the same thing, cancellation of the attraction between DPN^+ and $ZnOH^-$ in the former case by imidazole substituting OH^-, in the latter by H^+ neutralizing it.

In the "backward" reaction, imidazole accelerates the reaction at high [DPN] and high [alc] 5 to 10-fold in agreement with the destabilization factor $K_{EI,R}/K_{E,R}$ = 2.01/0.31 = 6.5.

At pH 9, the maximal acceleration caused by imidazole in the forward reaction was found to be 5.5-fold (44/8.1). In this case the destabilization factor $K_{EI,O}/K_{E,O}$ = 435/12 = 36. was much higher. The reason for this will be discussed below. In the backward reaction it was 4.5-fold (k_{16}/k_2 = 22/4.9) in perfect agreement with the destabilization factor ($K_{ER,I}/K_{E,I}$ = 3/0.67 = 4.5).

All the facts mentioned here concerning the influence of inhibitors, caprate, *iso*butyramide and imidazole (and alcohol!), on the dissociation constants and reaction velocities, conforms with the idea *that the entrance of a ternary ligand into a coenzyme-enzyme complex in most cases influences the "off" velocity but hardly at all the "on" velocity of the coenzyme.* Furthermore, the fact that the binding of both DPN and of DPNH to zinc is weakened by imidazole, in both cases resulting in stimulation of the reaction velocity, favours the suggestion of Wallenfels and Sund [3] that the adenine moiety which is equal in DPN and DPNH, is bound to Zn, rather than the pyridine ring, which is different in DPN and in DPNH. The amino group at C (6) and the N (7) was assumed

Table 15. Competitive features of the kinetics. Competition = +.

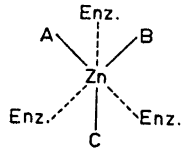
Case:	1	2	3	4
Caprate	+	−	−	+
*Iso*butyramide	−	+	+	−
Imidazole	−	+	+	+
o-Phenanthroline (Part IV)	+	−	+	±

to form a bidentate chelate with zinc. This forms a five membered ring, with zinc between two nitrogen atoms, and with two carbons, just as in ethylenediamine. Ethylenediamine as third bidentate ligand to zinc in octahedral bonds forms only weak complexes with association constants around 10^1, which is in agreement with the data for LADH + ethylenediamine reported in Part IV, and is also in agreement with the kinetic and equilibrium data for imidazole.

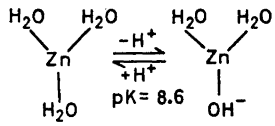
Along with the velocity constants the competitive features of the kinetics (Table 15) are illustrative. Caprate competed with DPNH and alcohol and the kinetic experiments, in agreement with the equilibrium data, showed that EI and EOI, but not ERI are formed. *Iso*butyramide was the opposite, being competitive with aldehyde and DPN, there being no real indication of effects due to the presence of EOI, only EI and ERI being formed. These differences in behaviour would indicate that alcohol and caprate have a different site of attachment compared with aldehyde and *iso*butyramide [1], and suggest the idea of different bonds for the substrates. Imidazole competes with both substrates and forms both the ternary complexes ERI and EOI. This behaviour of imidazole, a well known complexer with zinc, strongly suggests that zinc plays a central role in the binding of the substrates [3,4]. The formation by glycine of only the binary E-glycine, but no ternary coenzyme complexes in the equilibrium experiments of Part II, and the competition of *o*-phenanthroline (OP) and 2,2'-dipyridyl with DPNH and DPN (Part IV) show the importance of zinc attaching the coenzyme as bidentate ligands through the adenine moiety [3,4]. The mixed type of inhibition caused by o-phenantroline when alcohol (case 4) is varied will be discussed below (see p. 1862).

The structure of the binary and ternary complexes

The assumption of octahedral bond type with zinc attached to the protein by three coordinative valencies, and with three free from the protein was discussed in Part II. It explains why zinc is very firmly bound to the protein. Furthermore, the zinc is rigidly fixed at the protein, and the three free valencies directed to constant positions are not necessarily equivalent. They may be denoted A, B and C:



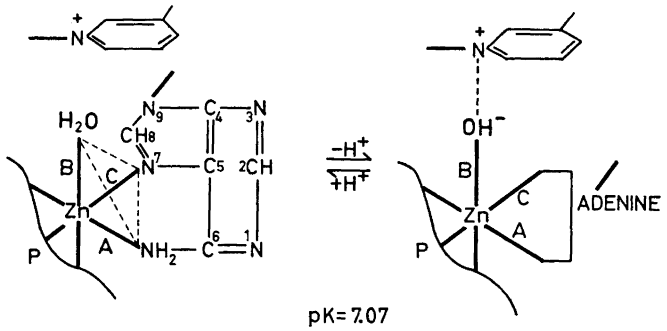
In neutral or acid solution the free valencies as usual carry water molecules, one or more of them losing protons at increasing pH:



As discussed in Part II, only one of the water molecules or hydroxo groups can be replaced by imidazole.

The attachment of DPN to zinc by the adenine moiety as bidentate ligand, with the pyridine ring close to the adenine (and hence to the zinc also, *i.e.* N^+ and OH^- in proximity) could be schematically represented as

LADH-DPN (EO)



(E)

$K_{E,O}$ = 288 μM (H_2O on bond B) $K_{E,O}$ = 8.5 μM
$K_{EI,O}$ = 440 μM (Imidazole on bond B) $K_{EI,O}$ = 440 μM

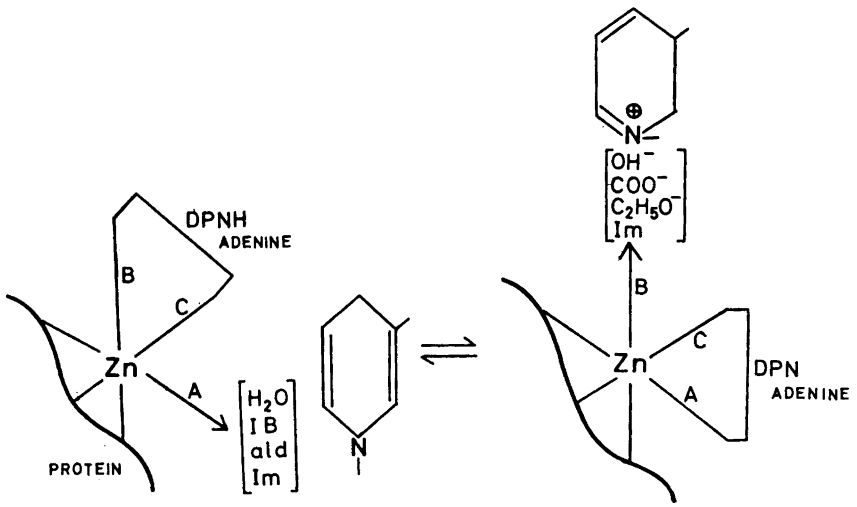
The introduction of imidazole at bond B instead of an hydroxo group abolishes the stabilization of the binary EO complex in alkaline solution, presumably caused by attraction between N^+ and OH^-. This is illustrated by the observation that at pH 9 the "off" velocity of DPN from EIO is only 5.3 times higher than that from EO. From the destabilization factor $K_{EI,O}/K_{E,O}$ = 440/12 one would have expected a 36—fold increase. The discrepancy could be explained if it is assumed that the coulombic attraction between $Zn—OH^-$ and N^+ (DPN), operating in the absence, but not in the presence of imidazole, would result in a higher "on" velocity in the former case. The increase would be required to be 36/5.5 = 6.5-fold.

As shown in Part II, $K_{EI,O}$ for caprate (7 μM) and long chain fatty acids was essentially the same as the alkaline asymptote value of $K_{E,O}$ (8.5 μM) which deponds upon the Zn — OH^- — N^+ interaction. Therefore there is reason to believe that the ternary EOI complexes with fatty acids have a similar structure with the carboxyl group interposed between Zn and the pyridine N^+, Zn — COO^- — N^+.

R

The strict competition between fatty acids and alcohol suggests that alcohol could be similarly attached as an alcoholate ion. The dissociation of the proton from alcohol which is required by the reaction mechanism receives an explanation as it must be favoured by the positive charge of pyridinium, and the interaction to the zinc. This effect will not operate with aldehyde or fatty acid amides, which do not ionize. Thus the positively charged pyridinium ring would seem sufficient to explain the formation of EOalc, and why the complexes EOald and EOamide are not formed.

The kinetic results, while substantiating the octahedral binding of zinc discussed above, also implicated it as the center of a ternary complex [3,4] where along with the three bonds to the protein and the two to adenine [3], the last now attaches the substrates. It is this bond which in the binary complex EO was joined to the pyridinium ring (Zn—OH^-—N^+) but in ER was occupied by a water molecule. It was also suggested previously that Zn in LADH was octahedral [5], but it was by some authors considered not to attach the substrates [6,7] or to be the center of a ternary complex [6]. Indeed zinc attachment to coenzyme pyrophosphate groups has been suggested [7,8]. although pyrophosphate does not affect the activity [9].



(F)

The ternary complexes and the interconversion phase

Since imidazole and caprate are both competitive with alcohol, bond B (scheme E) presumably can be used for attaching any of these. Aldehyde and *iso*butyramide, on the contrary, do not seem to be able to use this bond; the complexes EOIB, or EOald are not formed. Imidazole and *iso*butyramide in the kinetic experiments both compete with aldehyde, but there is no immediate reason why imidazole should not be able to use any one of the bonds A, B, or C. This brings up the question whether the adenine bonds could both move or one flick over ("edge shift") from A to B when DPN is reduced to DPNH, thus leaving bond A free for aldehyde or *iso*butyramide.

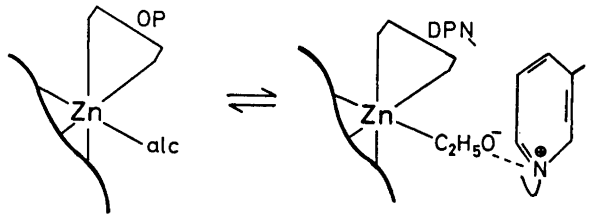
This hypothesis involves that during the rapid interconversion EOalc ⇌ ERald the substrate as alcohol uses bond B, as aldehyde bond A. Thus with DPN on the zinc bonds A and C, only alcohol, caprate or imidazole can compete at or go on the third bond B. DPN changing to DPNH implies bond shift so that A becomes free, which only accepts aldehyde, *iso*butyramide and imidazole. Thus as a result of a presumably small intramolecular rearrangement the coenzyme determines which of two bonds is available and so what can attach. This concept gains some support from the stereospecific requirement that due to their different structures the substrates must change their position somewhat relative to the enzyme surface [10]. However, while the competitive features of Table 15 generally agree with this model, it does imply that imidazole in case I should be competitive with DPNH (Figs. 9 and 13). With bond A saturated by a high [aldehyde], imidazole is prevented from attaching there, and therefore the imidazole should compete with DPNH for bonds B or C. This is not so, imidazole does not show competition with DPNH, and in agreement herewith ERimidazole complexes are formed (see Part II).

In the ternary complexes either negative or neutral ligands appear to be attached to the zinc in the vicinity of the DPN pyridinium or DPNH pyridine rings respectively. The charge interaction between N^+ and zinc makes alcohol much more negative in the complexes than in the free state as discussed on p. 1860. Imidazole, perhaps due to its conjugated structure, behaved in an intermediate way (see Part II). While R and IB mutually stabilized each other in the ternary complex, O and IB completely destabilized each other. Caprate behaved in the reverse way. Imidazole, however, caused partial destabilization with both R and O, but formed ternary complexes with both. This effect was parallelled in the competition experiments.

The competition experiments taken as a whole (Table 15) confirm that the three free zinc bonds are not used at random by the coenzymes, substrates and inhibitors. Restrictions on which bonds each can use obviously prevail, steric, charge forces or binding sites in addition to the bonds with zinc resulting in orientation on the enzyme surface. Caprate case 1 and IB case 3 (Figs. 1 and 7); caprate case 2 and IB case 4 (Figs. 2 and 8); caprate case 4, IB case 2, and imidazole case 2 and 4 (Figs. 4, 6, 10 and 12) are seen to be similar. In these cases there is either competition at the bond they can both attach at, or else they are not competing where one cannot attach.

The effect of bidentate ligands

The bidentate ligands, like *ortho*phenanthroline (OP), 2,2′-dipyridyl, ethylenediamine (or glycine, as indicated by the equilibrium data), are all strictly competitive with both DPN and DPNH, as would be expected when only three bonds are free and the coenzyme-adenine forms a bidentate itself. This confirms that the adenine bonds to Zn are essential for the activity. As these inhibitors would not be expected to have additional bonds to other sites, restrictions unless for steric reasons would not be expected, but independent of which two bonds out of three are used, competition with the adenine would result. Nevertheless it is possible that OP and the bidentate ligands might be only attaching at the coenzyme bonds. The lack of strict competition with both substrates and the nature of the competition between OP and the substrates which shows that EIald and EIalc (mechanism 1) must be formed, may indicate this. If OP and DPNH are restricted to use the same two bonds then in agreement with the experiments, case 2 would not be competitive. The mixed competition in case 4, might be influenced by a stabilizing effect of DPN^+ and $alcohol^-$ on each other ($N^+—alc^-—Zn$), in the equilibrium



(G)

increasing [alc] shifting the equilibrium to the right, thus simulating a certain degree of competition between alc and OP. If OP would be able to compete at any two bonds, competition or mixed competition would be expected in both cases 2 and 4. The experiments with OP and the bidentate inhibitors are fortunate in giving excellent support for the necessity that the coenzymes be attached as bidentates to zinc.

LADH thus emerges as a protein where zinc is held by three bonds, water molecules being on the other three zinc bonds. In the binary complexes two of these water molecules are replaced by bidentate ligand inhibitors or by the coenzyme adenine moeity acting as a bidentate ligand, the state of the remaining water molecule depending on the pH, it being more dissociated in EO than in ER. In the ternary complexes this latter water molecule or OH^- is substituted by substrates or unidentate ligands (Fig. 18). In the binary and ternary complexes with DPN, there is an additional enzyme-coenzyme-pyridine ring attachment through the $N^+—X^-—Zn$ interaction. The great stability of the DPNH complexes may be caused by bond formation between enzyme SH-groups and the pyridine ring [2].

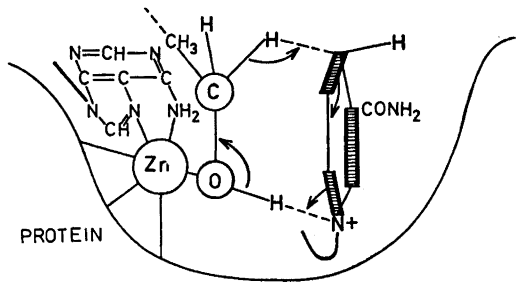


Fig. 18. Schematic representation of the LADH ternary complexes.

The theory presented here concerning how coenzymes and substrates are bound to Zn has the advantage of giving a possible explanation of how enzymes make coenzymes and substrates to select with great preference their active reaction partners, and reject those like ER alc, EO ald with which no reaction can occur.

The assumed shift of bonds (edge shift) from adenine to Zn on oxidoreduction of both coenzymes and substrates requires some shift in the conformation of the molecules, which one can easily imagine to take place with the coenzymes but less easily with the substrates. However, weak bonds and small displacements on the enzyme surface are probably involved.

While it is easy to envisage the positive charge of the pyridine ring in DPN being strong enough to bring about great preference for ligands with negative charge, e.g. fatty acids and alcohols, which may at least partly react as alcoholate ions, it is not so easy to visualize why aldehydes should be prevented from using the same bond, or why the neutral pyridine ring in DPNH should prefer aldehydes and amides. However, a zinc atom and the charge atmosphere around it would be expected to be very different in bonding or complexing properties to negative, neutral or conjugated molecules when a positive charge was present or absent in proximity, and of course even small differences in geometry and charge may be of importance in the crowded neighbourhood of the Zn atom in a ternary complex.

The abortive ERalc complex formed at very high alcohol concentrations may represent alcohol attached to the aldehyde zinc bond. The complex ERalc, which according to Winer and Theorell [1] should not be formed at all, shows that the formation of unreactive ternary complexes with both coenzyme and substrate is difficult, but not always entirely impossible. The specificity of fatty acids for EO, amides for ER may also be relative rather than absolute.

The inhibition of the reaction rate by very high concentrations of alcohol has now found an obvious explanation. Alcohol is the substrate in the first phase, an inhibitor in the second, acting there just as isobutyramide in forming a ternary complex, from which DPNH is dissociated more slowly than from DPNH-LADH.

The remarkable effect of imidazole stimulating the reaction velocity manifold at high substrate concentrations through the formation of labile ternary complexes in the second phase of the reaction, but inhibiting at lower substrate concentrations by effects in the first phase, could have a physiological significance. Substances of this kind would have a homeostatic function to maintain substrates at a suitable concentration. An excess would be rapidly removed, but the process would be strongly inhibited when lower concentrations were reached.

Note added to the proof December 16, 1961 by Hugo Theorell. An alternative theory which in some ways fits better with the experimental data is the following.

The zinc atoms in the free enzyme are held by three bonds to the enzyme, as assumed before. All other numbers than three are in fact controversial with experimental data. But in the free enzyme and in the binary complexes with monodentate ligands the arrangement could be *tetrahedral* instead of octahedral, leaving one bond free for attaching water, OH^-, imidazole, aldehydes, alcohols, amides or fatty acids.

This would explain why pH changes give a monovalent dissociation curve ($pK = 8.6$) for the free enzyme, and why only one molecule of imidazole can be attached, also giving a monovalent dissociation curve. $K_{E,Im,corr} = 0.6$ mM corresponds to an association constant = 3.2 which is not far from the value $k^4 = 2{,}65$ for free zinc ions in equilibrium with imidazole given in the literature (Part II, Ref. 3 b). Some difference must of course be expected from the probability that some of the three enzyme-zinc bonds are not imidazole residues.

Monodentate ligands which unlike water, hydroxo- or imidazole, are attached to the protein by additional groups, for example the lipofilic binding site operating with alcohols, aldehydes, fatty acids or amides may introduce some strain on the zinc bond, bending it somewhat in one or the other direction. The assumption of tetrahedral configuration with monodentate ligands readily explains why complexes with both imidazole and alcohol or aldehyde were not formed (*cf.* p. 1849), and why imidazole competes with both. On the other hand this theory would not allow the complexes E ald, caprate or E alc, *iso*butyramide to be formed.

Certain bidentate ligands like 1:10-phenanthroline and 2,2′-dipyridyl readily give quite strong octahedral complexes with zinc. It might therefore be possible that bidentate ligands can change the Zn bonds from tetrahedral to octahedral in LADH. The ternary complexes of LADH would therefore consist of one tridentate (LADH), one bidentate (adenine moiety of coenzymes or bidentate inhibitors) and one monodentate ligand (substrates or monodentate inhibitors).

Bidentate inhibitors forming octahedral complexes compete with the bidentate adenine of the coenzymes (*cf.* 1,10-phenanthroline in Part IV). Monodentate inhibitors compete with the substrates: imidazole competes with both alcohol and aldehyde, but not with DPNH. The competitive pictures between DPN and imidazole are caused by the inductive effect of the positively charged pyridine ring (*cf.* p. 1859). Aldehydes and amides by virtue of their lipofilic binding sites could distort the direction of the two bonds appearing on transformation to octahedral configuration enough to fit only the adenine moiety of DPNH; and alcohols or fatty acids — both negatively charged — could make them fit instead to the adenine of DPN. This direction change obviously substitutes the more complicated assumption of an "edge shift".

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REFERENCES

1. Winer, A. D. and Theorell, H. *Acta Chem. Scand.* **14** (1960) 1729.
2. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955). 1148.
3. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 59.
4. Mahler, H. R. and Douglas, J. *J. Am. Chem. Soc.* **79** (1957) 1159.
5. Vallee, B. L. and Coombs, T. L. *J. Biol. Chem.* **234** (1959) 2615.
6. Vallee, B. L., Williams, R. J. P. and Hoch, F. L. *J. Biol. Chem.* **234** (1959) 2621; Vallee, B. L. *The Enzymes, Vol. 3*, Academic Press 1960 p. 247.
7. van Eys, J., Pietro, A. San and Kaplan, N. O. *Science*, **127** (1958) 1443.
8. van Eys, J., Ciotti, M. M. and Kaplan, N. O. *Biochim. Biophys. Acta* **23** (1957) 574, 581.
9. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 48.
10. Gierer, A., *Biochim. Biophys. Acta* **17** (1955) 111.

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