ACTA CHEMICA SCANDINAVICA 15 (1961) 1797–1810

Liver Alcohol Dehydrogenase

I. Kinetics and Equilibria without Inhibitors

HUGO THEORELL and JOHN S. McKINLEY-McKEE

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

Liver alcohol dehydrogenase can form all the four binary complexes possible with DPNH, DPN, aldehyde and alcohol. The ternary complexes E-DPNH-aldehyde and E-DPN-alcohol are formed and are in very rapid equilibrium with one another. From a kinetic point of view the "Theorell-Chance" mechanism was nevertheless found to obtain. This depends on the following conditions:

1. The "on" velocity constants for DPNH and DPN are nearly independent of whether the enzyme is free or already combined with the substrate reaction partner.
2. The ternary complexes are in rapid equilibrium.
3. The ternary complexes prefer to liberate the substrate first, because this is less tightly bound than the coenzyme. The dissociation velocity of the binary enzyme-coenzyme complexes in the last phase of the reaction therefore becomes rate limiting when both substrate and coenzyme are present in high concentration in the first phase.
4. Determinations of initial reaction velocities with varied concentrations of both coenzyme and substrates have given Dalziel Φ-relations which agree with the specific requirements of the T.-C.-mechanism at pH 7

The values of the Michaelis constants close to zero concentration of their reaction partner, gave thedissociation constants $K_{E,R}$, $K_{E,O}$, $K_{E,ald}$ and $K_{E,alc}$.

The reaction mechanism of the liver alcohol dehydrogenase-coenzyme-substrate system * proposed in 1951 by Theorell and Chance [1] has been subject to repeated attempts at experimental confirmation [1-4]. In general, fair agreement was found between the values for the dissociation constants of the

* Abbreviations:

LADH and YADH:	Liver and yeast alcohol dehydrogenase, respectively
E:	1/2 LADH (since each molecule of LADH has 2 independent binding sites)
R:	reduced diphosphopyridine nucleotide, DPNH
O:	oxidized » » , DPN
S:	aldehyde
S':	alcohol

binary complexes ER and EO ($K_{E,R}$ and $K_{E,O}$) as determined from fluorometric equilibrium determinations in absence of substrate, and those calculated from the relation between the kinetic "off" and "on" rate constants, as determined in kinetic experiments with the substrates, assuming the T.-C. mechanism to be valid.

However, in connection with inhibition studies to be reported in Parts II and III of this series the opportunity arose of making decisive experiments to ascertain how far former disagreements, where there were indications that the formation or interconversion of the ternary complexes was perhaps effecting the rate [3,4], were occasional or not. We now had the advantage that the fluorometric titration of E with R in the presence of excess *iso*butyramide, enabled us to make very accurate determinations of the enzyme concentration.

Dalziel [5] has derived relations which distinguish the T.-C.-mechanism from other possible alternatives. This test necessitates carrying out series of kinetic experiments with submaximal concentrations of the constant reaction partner in each series. Four types of experiments are used for the determinations of the initial velocity, v ($\mu M \cdot sec^{-1}$).

	Constant	Varied
Case 1:	S	R
Case 2:	R	S
Case 3:	S′	O
Case 4:	O	S′

The results for each case are plotted according to Lineweaver and Burk [6] in terms of e/v against 1/[varied partner].

For the T.-C.-mechanism,

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER$$

$$ER + S \underset{k_3'}{\overset{k_3}{\rightleftharpoons}} EO + S'$$

$$EO \underset{k_1'}{\overset{k_2'}{\rightleftharpoons}} E + O$$

a steady state treatment gives the initial rate equations

$$\text{Case 1 and 2: } \frac{e}{v} = \frac{1}{k_2'} + \frac{1}{k_1[R]} + \frac{1}{k_3[S]} + \frac{k_2}{k_1 k_3[R][S]} \quad (1a)$$

$$\text{Cases 3 and 4: } \frac{e}{v} = \frac{1}{k_2} + \frac{1}{k_1'[O]} + \frac{1}{k_3'[S']} + \frac{k_2'}{k_1' k_3'[O][S']} \quad (1b)$$

(1a) and (1b) are the special T.-C. mechanism forms of Dalziel's general equation [5]:

$$\frac{e}{v} = \Phi_0 + \frac{\Phi_1}{S_1} + \frac{\Phi_2}{S_2} + \frac{\Phi_{12}}{S_1 S_2} \qquad (2)$$

Experiments with as high as possible concentration of the constant partner will, by combination of all four cases, give values for all six rate constants. However, in this case the Φ_{12}-term is always neglected. This is permissible in cases 1 and 2, where Φ_{12} is low, but not in cases 3 and 4, where Φ_{12} is high. Furthermore, as Dalziel has pointed out, determinations of e/v with submaximal concentration of the constant reaction partner can give independent values for the Φ_{12}-term, which are very important as a check of the T.-C.-mechanism. Experiments have therefore been carried out with both maximal and submaximal concentrations of the constant partner at pH 7, and with maximal concentrations at pH 9.

Eqn. 2 on rearrangement gives

$$\text{Case 1:} \quad \frac{e}{v} = \Phi_0 + \frac{\Phi_2}{[S_2]} + \left(\Phi_1 + \frac{\Phi_{12}}{[S_2]}\right)\frac{1}{[S_1]} \qquad (3a)$$

$$\text{Case 2:} \quad \frac{e}{v} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \left(\Phi_2 + \frac{\Phi_{12}}{[S_1]}\right)\frac{1}{[S_2]} \qquad (3b)$$

Due to the symmetry of the T.-C.-mechanism the introduction of primed symbols represents cases 3 and 4 for the reverse reaction. Thus in experiments where both substrates are present in concentrations low enough to be rate limiting (submaximal concentrations) [5,7], when (for various S_2 concentrations) S_1 is varied, plots of the intercepts (taken from the case 1 Lineweaver-Burk plots) against $1/S_2$ will give Φ_0 as an intercept and Φ_2 as a slope, while the slopes when plotted will give Φ_1 as intercept, and Φ_{12} as slope. When S_2 is varied (Case 2) the intercepts again give Φ_0 but with Φ_1 as slope, while the slopes give Φ_2 as intercept, with Φ_{12} as slope again. Cases 1 and 2 for the forward reaction thus give two values each for Φ_0, Φ_1, Φ_2, and Φ_{12}. Cases 3 and 4 likewise give Φ'_0, Φ'_1, Φ'_2, and Φ'_{12}. For the T.-C.-mechanism the identity of eqns. 1 and 2 for the forward and reverse reactions, give the relations $\Phi_0 = \Phi'_1\Phi'_2/\Phi'_{12}$, and $\Phi'_0 = \Phi_1\Phi_2/\Phi_{12}$, which are a sensitive way of distinguishing the T.-C.-mechanism from other mechanisms conforming to eqn. 2. Several of these mechanisms give the relation $K_{eq} = \Phi_{12}/\Phi'_{12}$, but because of the distinguishing Φ relations above, the T.-C.-mechanism has the further characteristic relation, but less sensitive test, $K_{eq} = \Phi_0\Phi_1\Phi_2/\Phi'_0\Phi'_1\Phi'_2$. It is these Dalziel relations [5] that have been tested at pH 7.

At 0.1 μ and 23.5°C the overall equilibrium constant $K_{eq} = [S_1][S_2]/[S'_1][S'_2] = 0.9 \times 10^{-4} \times [H^+]$ and therefore the forward reaction is greatly favoured from the thermodynamic point of view [8]. One would think this would lead to technical difficulties in cases 3 and 4, where the equilibrium is already reached when a very small part of [O] has reacted with [S']. However, in fluorometric determinations rather the opposite is true. In cases 1 and

2 we observe the disappearance of the fluorescence of R, and the whole reaction cycle is limited to the width of the paper, so that bent curves are often obtained. Furthermore, the highest [R] that can be used is limited by the extinction of the exciting light through absorption.

In cases 3 and 4 highest sensitivity and very small enzyme concentrations can be used, because only the very beginning of the reaction is of interest. A production of 10^{-7} M R is sufficient for determining the slope of the line, which will in this case be nearly straight. In addition, unlimited concentrations of O can be used, since it does not absorb the exciting light.

EXPERIMENTAL

Materials. DPN and DPNH were from the Sigma Chemical Co. Assay was carried out with a catalytic amount of YADH. Using an absorbancy index of 6.25×10^6 cm²/mole at 340 mμ [9], the βDPNH purity was 70—71 % by weight, 95 % on the basis of E_{340}, while the residual extinction after enzymatic oxidation was 5 % and the residual fluorescence which was corrected for was 6 %. Fig. 1 shows the relation between the concentration of DPNH solutions and their fluorescence. Quenching by absorption of the exciting light is seen and consequently concentrations above 13 μM where the correction is 8 % were seldom used. However, corrections were made where necessary, these being 6 % at 10 μM and 16 % at 26 μM. As DPNH is unstable in water or below pH 7.5, particularly when exposed to U.V. light, a stock DPNH solution was made in 0.01 M tris(hydroxymethyl)aminomethane, pH 10.6, and kept at 2°C in the dark. More dilute solutions were made with distilled water and kept at 2°C, except during actual use, when they were kept in the dark at 23.5°C. Stock solutions of concentration 1 050 μM (1 mg/ml) or greater were sometimes kept for periods of up to 30 h, but never longer or at lower concentrations, as otherwise decomposition become measurable. On the basis of an absorbancy index [10] of 18.0×10^6 cm²/mole, the βDPN purity was found to be 90—91 % by weight, and 99—100 % from the absorbancy at 260 mμ. Solutions (pH 4—7) were made in distilled water. Unless when in use, they were kept at 2°C in the dark where they were found to be stable, solutions of 1 360 μM (1 mg/ml) or more not changing for many days.

LADH was prepared from a horse liver according to Dalziel [11]. After chromatography on the carboxymethylcellulose column, the main component was twice crystallized in the presence of 30 % ethanol at −14°C and then 3 or 4 times recrystallized by dialysis against 6 % ethanol at 2°C [11]. The LADH crystals were kept at −14°C under 0.1 μ phosphate buffer, pH 7, containing 30 % ethanol. No measured or noticeable change was seen in the solutions prepared from the crystals over a two year period. Solutions were prepared by centrifuging some of the stirred suspension (~ 10 mg/ml) at 15 000 r.p.m. and −14°C, discarding the supernatant and dissolving the crystals in the same volume of 0.1 μ phosphate-ammonia buffer, pH 9. A clear solution resulted which was dialysed for 3—4 days against 0.1 μ phosphate pH 7 or 8, the buffer being changed each 12 or 24 h. Greatest stability to dialysis or standing was found between pH 8 and 9. Finally the solution was centrifuged at 15 000 r.p.m. and 2°C. Stock solutions resulted (~ 10^{-4} M) which were stable for many days; indeed on occasion the decrease in activity was less than the slight increase due to concentration by evaporation, although the enzyme was kept in a 3 ml stoppered glass bottle at 0°C. Dilute solutions were made, using either 0.1 μ phosphate buffer, pH 7 or 8. These were assayed immediately before and after an experiment, particularly when more dilute than 10^{-6} M. All enzyme solutions were maintained at 0°C or 2°C.

Enzyme solutions were assayed according to Dalziel [11,12], the enzyme concentration, $e = 1.13/(V)(t_{0.2})(84\ 000)$ moles/litre, where V is the volume (ml) of enzyme pipetted into the 3 ml assay solution (1.0 ml of 1 mg/ml DPN solution + 150 μl 0.16 M ethanol + 1.85 ml 0.1 M glycine-NaOH buffer, pH 10) in a quartz 1 cm cuvette, and t the time in seconds for the absorbancy at 340 mμ to increase by 0.2 units. The enzyme concentration used, however, was always 0.83 of this (2×0.83 μN) as in the spectrophotofluorometer the ratio of the binding sites concentration determined using excess *iso*butyramide to the μN concentration determined in the above spectrophotometric assay was 0.83. The enzyme

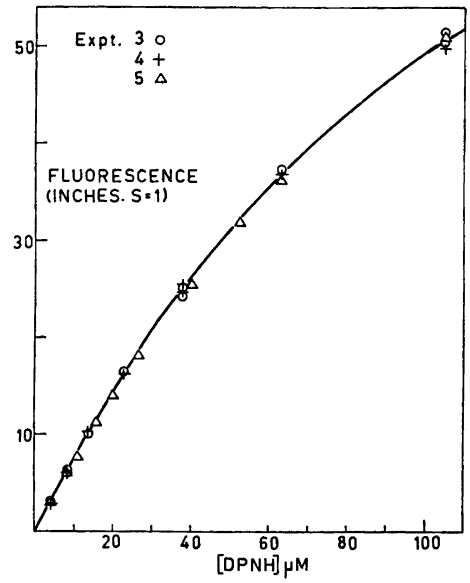


Fig. 1. Relation between the concentration of DPNH solutions and their fluorescence. Fluorimeter I. (The fluorescence has been corrected for a cuvette fluorescence of 0.1″. S = 1.)

was considered pure as stock enzyme solutions had an E_{280} which in relation to the activity had a specific extinction (1 mg/ml) of 0.41 – 0.42. The binding site concentration (as determined by titration with DPNH in the presence of *iso*butyramide) gives a specific extinction near 0.5.

All solutions were made in quartz redistilled water. This showed no fluorescence even at the highest sensitivity of the fluorometer, where there was, however, a background deflection of 0.1″ (S = 1.0) from the cuvettes. Buffer solutions were made with "analar" or "pro analysi" chemicals. At pH 7, 0.1 μ phosphate was used, while at pH 9, 0.1 μ Na_2HPO_4 + 3.6 mM glycine-NaOH buffer was used. Versene was never added to any solutions. 95 volume % alcohol ("finsprit") and Merck, Darmstadt, "puriss" acetaldehyde were distilled through 20″ Fensky helix packed columns and the middle third collected. They were kept apart at −14°C in the dark. Stock solutions were made in water by adding 200 μl, and checked by weighing and enzymatic assay. 95 volume % alcohol (5 % water) was used, as absolute alcohol on occasion contained traces of benzene.

Methods. The apparatus was essentially as described earlier [13]. A Luma Hg 125 water-cooled lamp was employed to excite the solutions with the 366 mμ mercury line, and very high sensitivity obtained by using wide entrance and exit slits (6 mm), thus enabling substrate concentrations down to 0.5 μM to be used. The LADH concentration was made low enough to give linear initial velocities. The enzyme (2 – 20 μl) and small substrate volumes (5 – 200 μl) were added from Carlsberg pipettes checked by the weight of clean mercury they held, and then calibrated from the absorption at 350 mμ of the amount of sodium dichromate they delivered, using the method of equal dilutions and calibrated volumetric glassware. Substrate solutions were kept in the dark either in the refrigerator or at 23.5°C when in immediate use. With the buffer they were added to give a final volume of 4 ml in 4.5 ml, 1 × 1 cm fluorescence- free glass cuvettes which when checked gave the same velocities as similar quartz cuvettes. pH (± 0.02) was measured at 23.5°C on a Beckman model G pH meter with microelectrodes. The enzyme was added from a glass stirrer in the dark with rapid stirring. Due to the non-fluorescence of DPN and the bluish white fluorescence of DPNH [14] the rate of DPNH oxidation or DPN reduction (e/V) can be measured by the rate of formation or disappearance of DPNH; $e/V = e\,\Delta D/(V_p \cdot S \cdot [D])$, where e = binding site enzyme concentration; V_p = recorder pen velocity-inches/sec.; S = sensitivity variable from 0.1 to 5; $[D]$ = concentration of the DPNH standard and

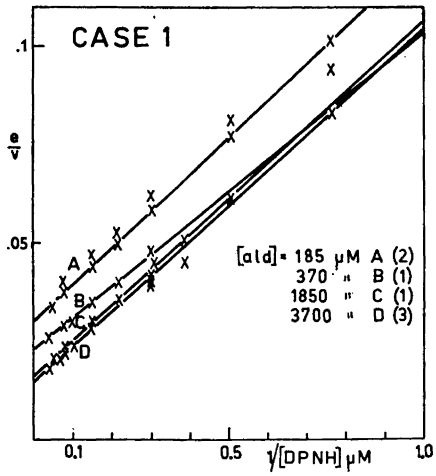


Fig. 2. Lineweaver-Burk plots for case 1. (The number of experiments on which each plot is founded is marked beside the particular plot designations.)

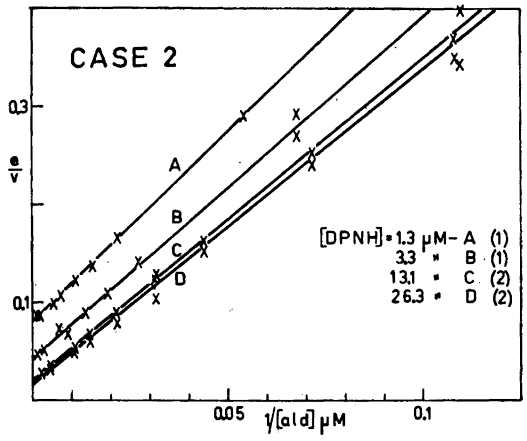


Fig. 3. Lineweaver-Burk plots for case 2.

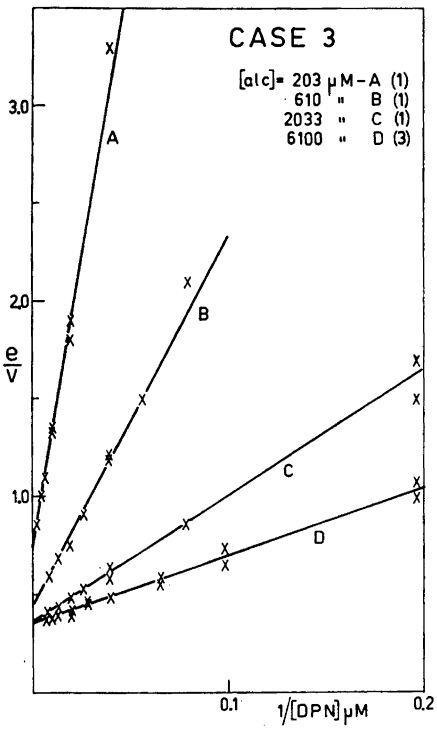


Fig. 4. Lineweaver-Burk plots for case 3.

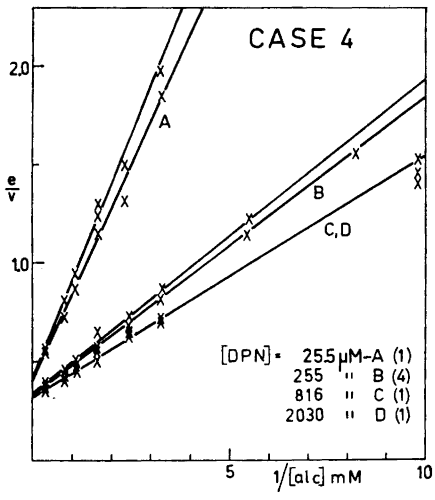


Fig. 5. Lineweaver-Burk plots for case 4.

Table 1. Φ-values and rate constants, pH 7.

Φ-values determined experimentally	Rate constants *
$\Phi_0 = 0.0135$ (0.014; 0.013) sec	$k'_2 = 74$ sec^{-1}
$\Phi_1 = 0.090$ (0.089; 0.091) sec $\times$ μM	$k_1 = 11.1$ sec^{-1} $\times$ μM^{-1}
$\Phi_2 = 3.2$ (3.2; 3.2) sec $\times$ μM	$k_3 = 0.31$ sec^{-1} $\times$ μM^{-1}
$\Phi_{12} = 0.9$ (0.96; 0.8) sec $\times$ μM^2	
$\Phi'_0 = 0.32$ (0.325; 0.32) sec	$k_2 = 3.12$ sec^{-1}
$\Phi'_1 = 1.9$ (1.9; 2.3) sec $\times$ μM	$k'_1 = 0.525$ sec^{-1} $\times$ μM^{-1}
$\Phi'_2 = 82$ (82; $\approx$ 110) sec $\times$ μM	$k'_3 = 0.0122$ sec^{-1} $\times$ μM^{-1}
$\Phi'_{12} = 10\,500$ (10 000; 11 000) sec $\times$ μM^2	

* Calculated from Φ values ($1/\Phi$).

Table 2. Equilibrium constants obtained from kinetics and equilibrium data, pH 7.

	Kinetic	Equilibrium
$K_{E,R}$	$k_2/k_1 = 0.28$ μM	0.31 μM *
$K_{E,O}$	$k'_2/k'_1 = 141$ μM	160 μM *
$K_{E,O}/K_{E,R}$	502	515
K_{eq}	$k_2k'_3k'_1 \times 10^{-7}/k'_2k_3k_1 = 0.78 \times 10^{-11}$ M.	0.90×10^{-11} M (Ref.[8])

* Part II.

Table 3. Relations between coefficients required by the Theorell-Chance mechanism according to Dalziel[5], pH 7.

$\Phi'_0 = \frac{\Phi_1\Phi_2}{\Phi_{12}}$	$\Phi'_0 = 0.32$ sec	$\frac{\Phi_1\Phi_2}{\Phi_{12}} = 0.32$ sec
$\Phi_0 = \frac{\Phi'_1\Phi'_2}{\Phi'_{12}}$	$\Phi_0 = 0.0135$ sec	$\frac{\Phi'_1\Phi'_2}{\Phi'_{12}} = 0.0149$ sec
$K_{eq} = \frac{\Phi_{12} \times 10^{-7}}{\Phi'_{12}}$	$K_{eq} = 0.90 \times 10^{-11}$ M	$\frac{\Phi_{12} \times 10^{-7}}{\Phi'_{12}} = 0.86 \times 10^{-11}$ M
$K_{eq} = \frac{\Phi_0\Phi_1\Phi_2 \times 10^{-7}}{\Phi'_0\Phi'_1\Phi'_2}$	$K_{eq} = 0.90 \times 10^{-11}$ M	$\frac{\Phi_0\Phi_1\Phi_2 \times 10^{-7}}{\Phi'_0\Phi'_1\Phi'_2} = 0.78 \times 10^{-11}$ M

ΔD its deflection in inches at $S = 1$. It was unaffected by direct exposure to the incident light for 5—10 min, a period much longer than the total time it was irridated during a series of experiments, when it was measured along with each cuvette, each exposure taking 3—4 sec. (Recently a "pure" βDPNH Sigma sample was found unsuitable in this respect as it was unstable to UV-light, ΔD decreasing during the course of an experiment). At the end of each experiment the standard was checked against unused standard taken from the refrigerator and carefully brought to 23.5°C. The fluorescence intensity of a DPNH solution changes noticeably with a 1° temperature change.

Table 4. Φ-values and rate constants, pH 9.

Rate constants	Φ-values *
k_2' = 8.15 sec^{-1}	Φ_0 = 0.122 (Φ_0 expt. = 0.122) sec
k_1 = 5.7 $sec^{-1} \times \mu M^{-1}$	Φ_1 = 0.175 sec $\times$ μM
k_3 = 0.062 $sec^{-1} \times \mu M^{-1}$	Φ_2 = 16.1 (Φ_2 expt. = 16.8) sec $\times$ μM
$\frac{k_2}{k_1 \times k_3}$ = 13.8 $sec^{-1} \times \mu M^{-2}$	Φ_{12} = 13.8 sec $\times$ μM^2
k_2 = 4.9 sec^{-1}	Φ_0' = 0.204 sec
k_1' = 0.508 $sec^{-1} \times \mu M^{-1}$	Φ_1' = 1.97 sec $\times$ μM
k_3' = 0.00885 $sec^{-1} \times \mu M^{-1}$	Φ_2' = 113 sec $\times$ μM
$\frac{k_2'}{k_1' k_3'}$ = 1 810 $sec^{-1} \times \mu M^{-2}$	Φ_{12}' = 1 810 sec $\times$ μM^2

* Calculated from rate constants. ($1/k$).

Table 5. Equilibrium constants obtained from kinetic and equilibrium data, pH 9.

	Kinetic	Equilibrium
$K_{E,R}$	k_2/k_1 = 0.86 μM	0.65 μM*
$K_{E,O}$	$k_2'k_1'$ = 16 μM	12 μM*
$K_{E,O}/K_{E,R}$	18.6	18.5
K_{eq}	$\frac{k_2k_3'k_1' \times 10^{-9}}{k_2'k_3k_1}$ = 0.77 $\times$ 10^{-11} M	0.90 $\times$ 10^{-11} M (Ref.[8])

* Part II.

RESULTS

For pH 7, Figs. 2—5 show the Lineweaver-Burk plots (to avoid uncertainty many points, several of which are done in duplicate, are usually required) for cases 1—4, while Figs. 6—13 show the plots of the intercepts and slopes which give the Φ values and resultant kinetic and equilibrium constants in Tables 1—3. Table 3 shows that the sensitive requirements of the T.-C.-mechanism are excellently fulfilled. The maximum velocities, k_2 (cases 3 and 4) and k'_2 (cases 1 and 2) are seen to be twice the 1955 value [2], which could partly be due to the titrations with *iso*butyramide giving a correct knowledge of the concentration of enzyme binding sites, along with the extrapolation of the velocities to infinite concentration of the constant reaction partner. Tables 4 and 5 give the results for pH 9, where experiments with submaximal concentrations were only performed for case 1. The older method of calculation [2] was used with the modification that the values for k_2 and k'_2 were found by crosswise extrapolation to infinite concentrations. Excellent agreement was obtained between the k_2 values from cases 3 and 4, and between the k'_2 values from cases 1 and 2.

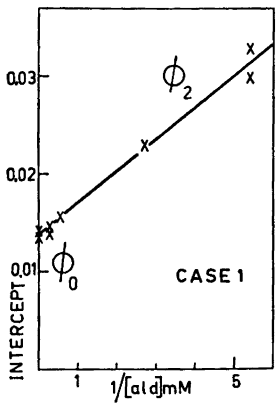


Fig. 6. Plot of intercepts, case 1.

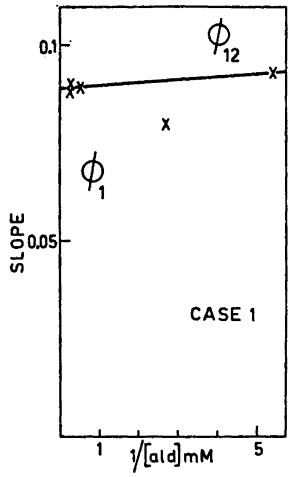


Fig. 7. Plot of slopes, case 1.

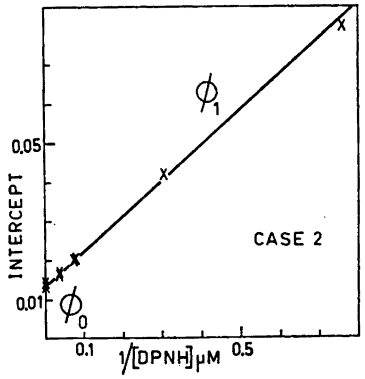


Fig. 8. Plot of intercepts, case 2.

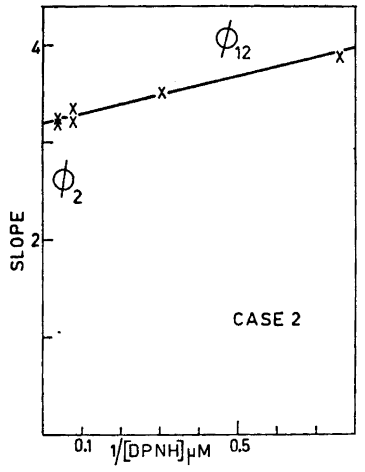


Fig. 9. Plot of slopes, case 2.

DISCUSSION

The results obtained at pH 7 and 9 give very convincing evidence that the T.-C.-mechanism obtains for the liver alcohol dehydrogenase system. All the criteria given by Dalziel agree with this mechanism. Of course, as already emphasized [1], the T.-C.-mechanism does not exclude the existence of binary ES and ES′ complexes or of the ternary complexes ERS and EOS′, dissociating and interconverting rapidly enough to not affect the maximum rate. Whether there are ternary complexes operating under these kinetic conditions or not,

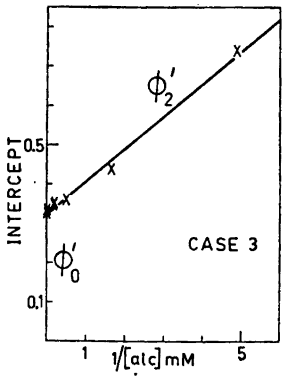


Fig. 10. Plot of intercepts, case 3.

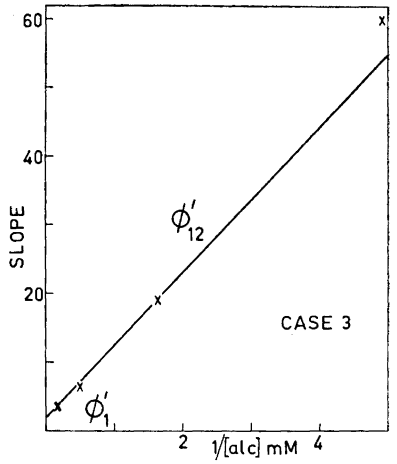


Fig. 11. Plot of slopes, case 3.

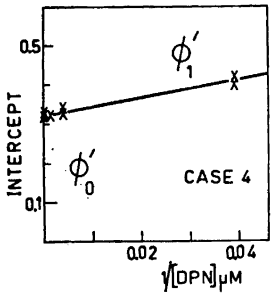


Fig. 12. Plot of intercepts, case 4.

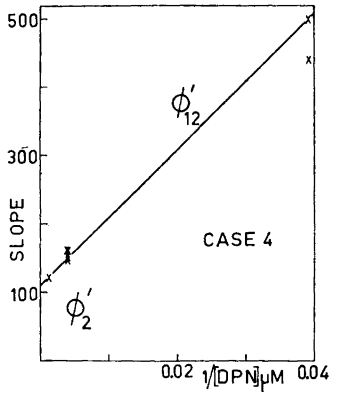
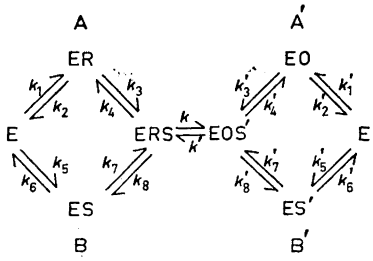


Fig. 13. Plot of slopes, case 4.

the present experiments could not indicate, although experiments where products were initially added might [15,16]. Indeed, in Part III we shall give experimental evidence for the existence of both types of complex, along with confirmatory values of the dissociation constants for ES and ES'. This means that the general mechanism I for an enzyme with two substrates is followed in the case of liver alcohol dehydrogenase, though with such restrictions that the T.-C. simplification is allowed at least at pH 7.



(I)

We can distinguish different phases in this reaction scheme: the first "on" phase and the second "off" phase. Between these interconversion operates. The reaction will prefer the routes in the four cases as shown in Table 6.

It is evident from Tables 2 and 5 that the dissociation constants, determined in equilibrium measurements without substrates (See Part II) agree with the values calculated from the kinetic constants, thus $K_{E,R,\,eq} = k_2/k_1 = 0.31\ \mu M$,

Table 6. Reaction pathways for cases 1—4.

Case	Conditions	First phase	Second phase
1	Constant [S] R → 0	B	A′
2	» [R], S → 0	A	A′
3	» [S′], O → 0	B′	A
4	» [O], S′ → 0	A′	A

$K_{E,O,\,eq} = k_2'/k_1' = 160\ \mu M$ and as shown below and in Part III, $K_{E,S} = 10.2\ \mu M$, $K_{E,S'} = 4.6$ mM and thus $K_{ER,S}/K_{ES,R} = K_{E,S}/K_{E,R} = 33$ and $K_{EO,S'}/K_{ES',O} = K_{E,S'}/K_{E,O} = 29$. Therefore it appears highly likely that as required by the T.-C.-mechanism the dissociation reactions of the ternary complexes in the second phase of the reaction will prefer the routes A and A′, thus

$$E\begin{matrix}R\\S\end{matrix} \rightarrow ER \rightarrow E + R \quad \text{and} \quad E\begin{matrix}O\\S'\end{matrix} \rightarrow EO \rightarrow E + R$$
$$\begin{matrix}+\\S\end{matrix} \qquad\qquad \begin{matrix}+\\S'\end{matrix}$$

In the reverse reaction, at [O] → ∞ and [S′] → ∞ practicaly all the enzyme will be in the state ER, and $V_{max} = k_2$. The kinetic determinations of V_{max} therefore will be expected to give k'_2 and k_2 rather than k'_6 and k_6, in agreement with the T.-C.-mechanism. The reaction in the first phase will follow the upper or lower routes depending on the relative concentrations of the reaction partners. The values calculated as k_1 (from case 1) and k'_1 (from case 3) will therefore rather reflect the "on" velocity constants k_7 and k'_7

for $ES + R \xrightarrow{k_7}$ and $ES' + O \xrightarrow{k'_7}$. According to Tables 2 and 5, $K_{E,R,eq} \approx K_{E,R,kin}$, and $K_{E,O,eq} \approx K_{E,O,kin}$, which means that $k_1 = k_7$ and $k'_1 = k'_7$, in agreement with an earlier discussion [17]. It is by no means obvious, why this should be so. However, as we shall show experimentally in Part III, there seems to be a more general rule saying that partners in a ternary complex do not seem to interfere appreciably with each other's "on" velocity constants, but mainly with the "off" velocity constants, resulting in mutual stabilization to the same extent as the "off" velocity decreases, or mutual repulsion resulting in an increased "off" velocity.

The interconversion phase is in both directions very fast compared with the other steps, as required by the T.-C.-mechanism.

As seen in Figs. 2—5 the Michaelis constants vary with the concentration of the other partner in opposite ways for the forward and reverse reactions. This is inherent in the T.-C. mechanism:

From equation (1a) at $R = \infty$

$$\frac{e}{V} = \frac{1}{k_2'} + \frac{1}{k_3[S]}$$

Therefore at $[DPNH] = K_M$

$$\frac{1}{k_2'} + \frac{1}{k_3[S]} = \frac{1}{k_1[R]} + \frac{k_2}{k_3[S] \times k_1[R]}$$

$$K_M = [R] = 1 + \frac{k_2}{k_3[S]} \Big/ \frac{k_1}{k_2'} + \frac{k_1}{k_3[S]}$$

This hyperbolic function $(1 + ax)/(b + cx)$ gives an asymptotic value for $K_M = k_2'/k_1 = 6.7$ μM for $[S] = \infty$, and $K_M = k_2/k_1 = 0.28$ μM for $[S] = 0$. K_M approaches infinity for the unreal condition $1/[S] = -k_3/k_2' = -0.0042$.

Table 7 shows the values of the Michaelis constants obtained by analogous considerations at infinite and zero concentrations of each reaction partner. In all cases there is a 23.8-fold relation, operating in the opposite way for the forward and reverse reactions. As can be seen from Table 7 this is k_2'/k_2 (74/3.1 = 23.8). The condition for K_M being independent of the concentration of the other partner is that $k_2' = k_2$ (which is not true in our case).

As seen from Table 7 the Michaelis constants for DPNH and DPN, when their reaction partners approach zero concentration, asymptotically become equal to the dissociation constants $K_{E,R} = k_2/k_1$ and $K_{E,O} = k'_2/k'_1$. This reflects the situation:

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER$$

When [E] = [ER] and [ald] is very low, the reaction velocity will be equal to half that recorded when $[R] \to \infty$, that is $[R] = K_{M,DPNH}$ and *vice versa* for $K_{M,DPN}$. This is as expected since the T.-C. mechanism assumes that binary coenzyme-enzyme complexes are formed before any reaction with the substrate occurs.

Table 7. Dependence of the Michaelis constants on the concentration of the other reaction partner [C].

	K_M (μM)	
	[C] → 0	[C] → ∞
DPNH	$k_2/k_1 = 0.28$	$k_2'/k_1 = 6.7$
Aldehyde	$k_2/k_3 = 10$	$k_2'/k_3 = 239$
DPN	$k_2'/k_1' = 141$	$k_2/k_1' = 5.9$
Alcohol	$k_2'/k_3' = 6\,060$	$k_2/k_3' = 255$

From the general two substrate mechanism A, one would expect *mutatis mutandis* $K_{M,ald} = K_{E,ald} = k_6/k_5$, and $K_{M,alc} = K_{E,alc} = k_6'/k_5'$ for [R] resp. [O] → 0, and the values found, 10 μM and 6 100 μM are close to those found (in Part II) for $K_{E,ald}$ (10 μM) and $K_{E,alc}$ (4 600 μM) in experiments ments with inhibitors. The functions k_2/k_3 and k_2'/k_3' given in Table 7 for $K_{M,ald}$ and $K_{M,alc}$ are valid only under the assumptions of the T.-C. mechanism, that no binary enzyme-substrate complexes (ES, ES') are formed. The results obtained here, however, prove that such complexes are formed in agreement with the general mechanism I. Nevertheless the relations between the rate constants are such that the T.-C. simplification of the general mechanism I is kinetically adequate.

Note added in proof. K. Dalziel [18,19] has recently reported that impurities such as adenosine-diphosphate-ribose present in some DPNH preparations may have an inhibiting action, leading to too low values for k_2'. His highest value [19] for k_2' at pH 7 was = 100 which is somewhat higher than our value 74. However, we have confidence in our value for two reasons: first, it has been obtained consistently with three different DPNH preparations; second, these preparations were thoroughly analyzed (McKee, to be published) and were found to be at least as pure as Dalziel's best one. The conformity of our data to the requirements of the T. C. mechanism, *e.g.* $\Phi_0 = \Phi_1'\Phi_2'/\Phi_{12}'$ (Table 3) could be to some extent fortuitous. It can in fact be shown that strict simplification of the complex initialrate equation [16] for the general mechanism I to the simple T. C. equations 1 (a) and 1(b) reqires contradictory assumptions for the forward and reverse reaction, but approximate simplification can under certain conditions be expected theoretically (Dalziel, in preparation).

Acknowledgements. The authors are indebted for financial support given by *Statens Medicinska Forskningsråd, Statens Naturvetenskapliga Forskningsråd, Knut och Alice Wallenbergs Stiftelse, The Rockefeller Foundation, Wenner-Grenska Samfundet* and *Institutet för Maltdrycksforskning.*

Skilful technical assistance has been given by Mr. Lars-Gunnar Falksveden.

REFERENCES

1. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
2. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
3. Dalziel, K. and Theorell, H. *Biochem. J.* **66** (1957) 34P; Theorell, H. *Advances in Enzymol.* **20** (1958) 471.

4. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
5. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 1706.
6. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
7. Alberty, R. A. *J. Am. Chem. Soc.* **75** (1953) 1928.
8. Bäcklin, K. I. *Acta Chem. Scand.* **12** (1958) 1279.
9. Rafter, G. W. and Colowick, S. P. *Methods in Enzymology,* **Vol. III,** p. 887. Academic Press, New York 1957.
10. Kornberg, A. and Pricer, W. E. *Biochem. Preparations* **3** (1953) 20; Siegal, J. M., Montgomery, G. A. and Bock, R. M. *Arch. Biochem. Biophys.* **82** (1959) 288.
11. Dalziel, K. *Acta Chem. Scand.* **12** (1958) 459.
12. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 397.
13. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877.
14. Warburg, O. and Christian, W. C. *Biochem. Z.* **287** (1936) 287.
15. Alberty, R. A. *J. Am. Chem. Soc.* **80** (1958) 1777.
16. Dalziel, K. *Trans. Faraday Soc.* **54** (1958) 1247.
17. Winer, A. D. and Theorell, H. *Acta Chem. Scand.* **14** (1960) 1741.
18. Dalziel, K. *Nature* **191** (1961) 1098.
19. Dalziel, K. *Biochem. J.* **80** (1961) 440.

Received March 28, 1961.