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Studies on Liver Alcohol Dehydrogenase

III. The Influence of pH and Some Anions on the Reaction Velocity Constants

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By the aid of fluorescence measurements the initial reaction velocity constants in the liver ADH ** system have been determined in the pH range 5.3 to 10. Our previously postulated reaction mechanism appears to be followed as indicated by three independent lines of evidence:

1. The kinetical data when applied for calculating the equilibrium constant K of the whole system using the equations derived from our postulate gave values fairly close to the true dissociation constant, 10^{-11}, in the whole pH range.
2. The relationship between the dissociation constants of the oxidized and the reduced enzyme · coenzyme complex agreed with previous equilibrium data with excess of enzyme.
3. Two of the velocity constants had previously been determined spectrophotometrically in single reaction steps and agreed with our present data from the overall reaction velocities.

On the basis of this reaction mechanism, the significance of the different velocity constants and their variations with pH has been discussed.

Different anions were found to have remarkable effects on some of the velocity constants. In some cases, these effects were of opposite sign for the oxidized and reduced enzyme complexes and the oxidation-reduction potential could be calculated to be strongly dependent on the anion concentration. The significance of these findings is discussed. Liver ADH has been found to contain two atoms of zinc per molecule.

In the previous papers of this series [1,2] some data were given for the velocity constants in the reversible system

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** *Abbreviations.* DPNH: reduced, and DPN: oxidized diphosphopyridine nucleotide; ADH: ½ molecule of alcohol dehydrogenase; ald: acetaldehyde; alc: ethanol; x, x^1, e, a and a^1: concentrations of corresponding reactants.

$$\overset{x}{\text{DPNH}} + \overset{e}{\text{ADH}} \underset{k_2\ (\text{sec}^{-1})}{\overset{k_1\ (\text{M}^{-1} \times \text{sec}^{-1})}{\rightleftharpoons}} \text{DPNH} \cdot \text{ADH} \qquad (1)$$

$$\text{DPNH} \cdot \text{ADH} + \overset{a}{\text{ald}} + \text{H}^+ \underset{k_6\ (\text{M}^{-1} \times \text{sec}^{-1})}{\overset{k_4\ (\text{M}^{-2} \times \text{sec}^{-1})}{\rightleftharpoons}} \text{DPN} \cdot \text{ADH} + \overset{a^1}{\text{alc}} \qquad (2)$$

$$\text{DPN} \cdot \text{ADH} \underset{k_5\ (\text{M}^{-1} \times \text{sec}^{-1})}{\overset{k_3\ (\text{sec}^{-1})}{\rightleftharpoons}} \overset{x^1}{\text{DPN}} + \text{ADH} \qquad (3)$$

It was shown that the experimental data were reasonably compatible with the reaction scheme 1—3. This scheme requires a compulsory order of reactions: the coenzyme first combining with the enzyme, and the complex then reacting with the substrate. Furthermore, if a ternary complex is formed, the dissociation velocity of the substrate product must be high compared with the dissociation velocity of the coenzyme-enzyme. The older data were neither accurate, nor extensive enough to warrant definite conclusions as to the validity of this reaction scheme. The problem could be settled only by more accurate determination of the six velocity constants, and the relationship between these and the equilibrium constant.

Alberty [3] has derived formulas for different cases of reaction sequences for systems consisting of enzyme, coenzyme and substrate. When inserting our values into his formulae he found, that some of the data fitted with the reaction scheme 1—3, but others did not. We have now shown that this discrepancy was mainly due to the inaccuracy of some older data and the uncertainties in the determinations of some Michaelis constants.

The spectrophotometric methods used in 1951 did not permit us to work at sufficiently low concentrations of the reactants or at high enough acidities to get a sufficiently complete survey of the influence of pH on the magnitude of the reaction velocity constants. This is now possible by using fluorimetry instead of spectrophotometry [4]. The strong anion effects on the association and dissociation reactions of coenzyme and protein in the old yellow enzyme (O. Y. E.) [5] prompted us to make a corresponding investigation of the ADH system.

MATERIALS AND METHODS

DPNH gives a bluish-white fluorescence [6] that is not quenched by coupling to ADH, whereas DPN does not fluoresce. It is thus possible to follow the oxidation of DPNH or reduction of DPN by fluorescence measurements.

The experiments were carried out in 1 cm cuvettes, in a total volume of 3 ml. The reactions were always started by adding a suitable amount of ADH from a stirring rod at $t = 0$. In our apparatus, the intensity of the fluorescence of DPNH was about 4 % of the fluorescence of flavin mononucleotide (FMN) on an equimolar basis. Still the sensitivity is very high compared to spectrophotometry. A micromolar solution of DPNH in 1 cm cuvettes, when using sensitivity 10 inches per 0.1 volts, entrance slit 1 mm, and exit slit 6 mm, gave deflections of around 5 inches on the recorder. With larger entrance slits the inflections increase proportionally. For a comparison with spectrophotometry it should be remembered that the light absorption of the same solution at 340 mμ would be as low as $\log I_0/I = 0.006$. The practical limit of the sensitivity of the apparatus is set by the

fluorescence of the buffer solutions. Because of the high sensitivity of the apparatus, it was feasible to determine much smaller amounts of ethyl alcohol or acetaldehyde than with spectrophotometry. From the rate of the oxido/reduction of DPN(H) in the ADH system we have been able to determine quantitatively concentrations of alcohol and acetaldehyde down to 0.5×10^{-6} M.

The fluorescence of DPNH is proportional to the concentration up to about 20×10^{-6} M. Above this concentration, the 340 mμ band absorbs the incident light appreciably, so that corrections must be made.

Crystalline ADH was prepared according to the method of Bonnichsen [7]. The concentration of ADH was determined both from optical density at 280 mμ, and from kinetic measurements. Thus, k_2 at pH 7.15, 23.5°, was found to be 1.6 sec^{-1} for a homogeneous preparation of ADH. The maximum rate for the oxidation of alcohol was consequently determined for all preparations under these conditions, and the concentration of ADH determined on the basis of $k_2 = 1.6$ sec^{-1}. There was always agreement between the kinetic and spectrophotometric measurements.

DPN: A preparation which was 75 % pure, was made according to Neilands and Åkeson [8] in this laboratory.

DPNH was produced enzymatically, as described by Bonnichsen [9]. The preparation was lyophilized.

Buffers. The remarkable effects of anions observed on the O.Y.E. system [5], prompted us to make a careful selection of buffers for studying the pH effects independent of anion effects. The buffers were tested at different concentrations to make sure that they did not interfere appreciably with the reaction velocity. 0.05 M citrate was chosen at pH 5.3, phosphate, ionic strength 0.1, at pH 6 and 7, 0.1 M glycyl-glycine at pH 8, and 0.1 M glycine at pH 9 and 10. It is to be noticed that 0.05 M pyrophosphate, which has been used in previous experiments [1,2], inhibits the reduction of acetaldehyde appreciably at pH 9, so that at this pH the older values are not strictly comparable to the new ones.

CALCULATIONS

We have in this investigation exclusively determined the initial reaction velocities at $t \sim 0$. The reaction scheme 1—3 was found [2] to lead to the following equations:

Reduction of aldehyde by DPNH:

$$\frac{1}{e} \times \frac{dc}{dt} = \frac{1}{\frac{1}{k_1 x} + \frac{1}{k_4 [H^+] a}\left((1 + \frac{k_2}{k_1 x}\right) + \frac{1}{k_3}} \qquad (4)$$

Oxidation of ethanol by DPN:

$$\frac{1}{e} \times \frac{dc}{dt} = \frac{1}{\frac{1}{k_5 x^1} + \frac{1}{k_6 a^1}\left(1 + \frac{k_3}{k_5 x^1}\right) + \frac{1}{k_2}} \qquad (5)$$

In order to determine all six velocity constants at a given pH, ion concentration and temperature it is necessary to make four sets of experiments varying the reactants according to Table 1.

In most circumstances it is possible to obtain a reaction velocity low enough to be practically constant during the first 30—60 seconds by making the ADH concentration suitably low (see Fig. 1). Fig. 2 shows a reaction cycle with high [ald] and low [DPNH] (= 1.0 μM). Here the enzyme concentration was unsuitably large for obtaining a reliable tangent to the curve at $t = 0$; but,

Table 1. Variation in the concentrations of the reactants.

Expt. No.	[DPNH]	[ald]	[DPN]	[alc]
1	Varied	High	—	—
2	High	Varied	—	—
3	—	—	Varied	High
4	—	—	High	Varied

as would be expected, the reaction under the conditions used was of first order, so that confirmation of the slope at $t = 0$ could be obtained from other points on the curve.

The results from the four series of experiments when plotted according to Lineweaver and Burk [10] gave straight lines within the limits of error. However, alcohol concentrations >10 mM caused inhibition and were avoided.

We used the following procedure for calculating the velocity constants.

Two independent values for k_3 were obtained from the experiments Nos. 1 and 2, Table 1. e/V'_{max} is the intercept of the Lineweaver-Burk plot with the ordinate. In order to obtain k_3, which is equal to V_{max}/e at [DPNH] $= \infty$ and [ald] $= \infty$ [2] the value for e/V'_{max} from expt. No. 1 was corrected to infinite [ald]. Thus

$$k_3 = \frac{e}{V'_{max}}\left(1 + \frac{K_{m,ald}}{[ald]}\right) = \frac{V_{max}}{e} \qquad (6a)$$

and correspondingly from expt. 2

$$k_3 = \frac{V''_{max}}{e}\left(1 + \frac{K_{m,DPNH}}{[DPNH]}\right) = \frac{V_{max}}{e} \qquad (6b)$$

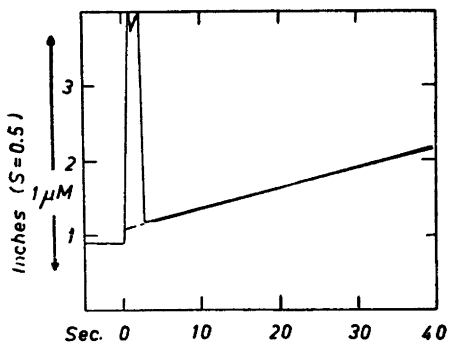


Fig. 1. Record of the increase in fluorescence after adding ADH at $t = 0$ to DPN and alcohol. pH 7.15. [ADH] $= 1.9 \times 10^{-8}$ M; [DPN] $= 4.8 \times 10^{-6}$; [alc] $= 7.7 \times 10^{-3}$. $S = 0.5$, slits 3 and 6 mm.

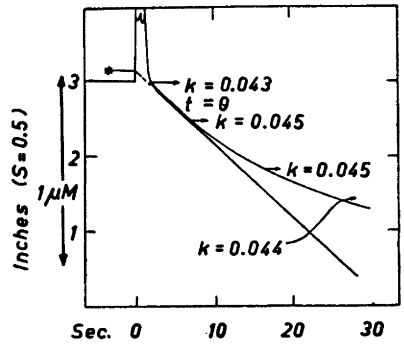


Fig. 2. Record of the decrease in fluorescence after adding ADH to DPNH and acetaldehyde. pH 7.15. ADH $= 1.65 \times 10^{-8}$ M; [DPNH] $= 1.0 \times 10^{-6}$, [ald] $= 3.8 \times 10^{-3}$.

k_2 is obtained from expts. 3 and 4 in an analogous manner. It should be noticed that this extrapolation to V_{max}/e is permissible only if the concentration of the reactant that is kept "high" and constant is considerably higher than K_m. This is also a prerequisite for obtaining approximately true Michaelis constants for the varied reactant.

We then chose a pair of points, one from each of the experiments 1 and 2, which gave the same e/V. The concentration of the aldehyde was then a_1 and a_2, the concentration of DPNH $= x_1$ and x_2. Under these conditions we found

$$k_4[H^+] = \frac{k_1 x_1 x_2 \ (a_1 - a_2) + k_2(a_1 x_1 - a_2 x_2)}{a_1 a_2 \ (x_1 - x_2)} \qquad (7)$$

Since k_2 is known the value for k_1 can thus be found from eq. 4, and k_4 $[H^+]$ from eq. 7.

k_5 and k_6 were obtained from experiments Nos. 3 and 4 (Table 1) in an analogous way. Many different constants can be derived from the velocity constants, as indicated by Table 2.

Table 2. Dependance of Michaelis and equilibrium constants on the kinetic constants, assuming the reaction sequence 1—3 to be valid.

Michaelis constants, K_m				Dissociation constants for holoenzyme			Equilibrium constant
							K
DPNH	ald	DPN	alc	Reduced D_{red}	Oxidized D_{ox}	D_{ox}/D_{red}	
$\frac{k_3}{k_1}$	$\frac{k_3}{k_4[H^+]}$	$\frac{k_2}{k_5}$	$\frac{k_2}{k_6}$	$\frac{k_3}{k_1}$	$\frac{k_3}{k_5}$	$\frac{k_3 k_1}{k_5 k_2}$	$\frac{k_2 k_5 k_6}{k_1 k_3 k_4}$

RESULTS

The new data are summarized in the Tables 3 and 4, and Fig. 3 shows the Lineweaver-Burk plots of some experiments in phosphate, phosphate + chloride and phosphate + formate at pH 7.15. We had now the possibility to check the validity of the proposed reaction scheme in the whole pH range 5.3 to 10.

Michaelis constants

It is seen from Tables 3—4 that the agreement between the experimentally observed values for K_m and the values calculated from the velocity constants is good in the experiments with DPNH and aldehyde. Greater deviations are found in the experiments with DPN and alcohol at pH 5.3 to 7.15. In some cases this is probably because the Michaelis constants are so high that the reactant which was held constant could not be used in great enough excess.

Table 3. Reaction velocity constants at 23.5° Ionic Strength of all buffers (Na-Salts) = 0.1

Velocity constants	pH 5.3 Citrate	pH 6.05 Phos-phate	pH 7.15 Phos-phate	pH 7.15 Phos-phate +0.15 M NaCl	pH 7.15 Phos-phate +0.015 M formate	pH 8.0 Glycyl-glycine	pH 9.0 Glycine	pH 10 Glycine
$10^{-6} \times k_1$	1.5	3.3	3.7	1.8	3.7	3.7	2	0.5
k_2	0.28	0.65	1.6	2.8	1.6	1.6	1.9	2
$10^{-6} \times k_4$ $[H^+]$	0.036	0.036	0.24	0.21	0.24	0.069	0.012	0.0008
$10^{-13} \times k_4$	0.00073	0.004	0.34	0.30	0.34	0.69	1.2	0.8
$10^{-4} \times k_6$	0.011	0.039	0.35	0.19	0.12	0.85	0.35	0.09
k_3	6	17	37	18	9.5	20	5	0.7
$10^{-6} \times k_5$	0.011	0.075	0.30	0.14	0.30	0.28	0.18	0.04

However, this is not likely to be the whole explanation of the discrepancies. Some deviation from the postulated mechanism appears to occur within this region. This deviation could be connected with the fact that alcohol in concentrations as low as 10 mM already has an inhibitory effect.

The new data for the K_m are in fair agreement with the older data with two exceptions; K_m for aldehyde has now been found to be 270 μM at pH 8, 400 μM at pH 9, whereas in the previous work we obtained 52 and 2 300, respectively. The difference may be due to the use of non-inhibiting buffers in the present experiments. In this connection it should be mentioned that for some unknown reason we had to repeat the experiments with aldehyde at pH 9 several times to get reliable values. The experimental data at pH 10 are definitely less certain than the others.

Table 4. Michaelis constants, equilibrium constants, and oxidation-reduction potentials calculated from kinetic constants.

Michaelis and equilibrium constants	pH 5.3	pH 6.05	pH 7.15	pH 7.15 NaCl	pH 7.15 NaCOONa	pH 8	pH 9	pH 10
k_3/k_1 μM	4.0	5.1	10	10	2.6	5.4	2.5	1.4
K_m, DPNH exptl. »	3.5	4.7	10	10	2.5	5.4	3	2.1
k_3/k_4 H^+ »	170	470	150	86	40	290	400	880
K_m, ald., exptl. »	270	410	110	50	41	270	400	800
k_2/k_5 »	25	9	5	20	5	5.7	10.5	50
K_m, DPN, exptl. »	77	25	10	33	10	5.0	11	40
k_2/k_6 »	2 700	1 700	460	1 500	1 330	190	550	2 200
k_m, alc., exptl. »	4 600	2 500	590	2 100	2 900	250	600	2 000
$K = \frac{k_2 k_5 k_6}{k_1 k_3 k_4} \times 10^{11}$	0.50	0.93	0.37	0.77	0.48	0.73	0.95	2.6
$D_{ox}(= k_3/k_5)$ μM	550	226	123	129	32	71	28	18
$D_{red}(= k_2/k_1)$ »	0.19	0.20	0.43	1.6	0.43	0.43	0.95	4
D_{ox}/D_{red} —	2 900	1 150	285	83	74	165	29	4.5
E_0 (holoenzyme), V	—0.126	—0.156	—0.204	—0.219	—0.221	—0.234	—0.290	—0.342

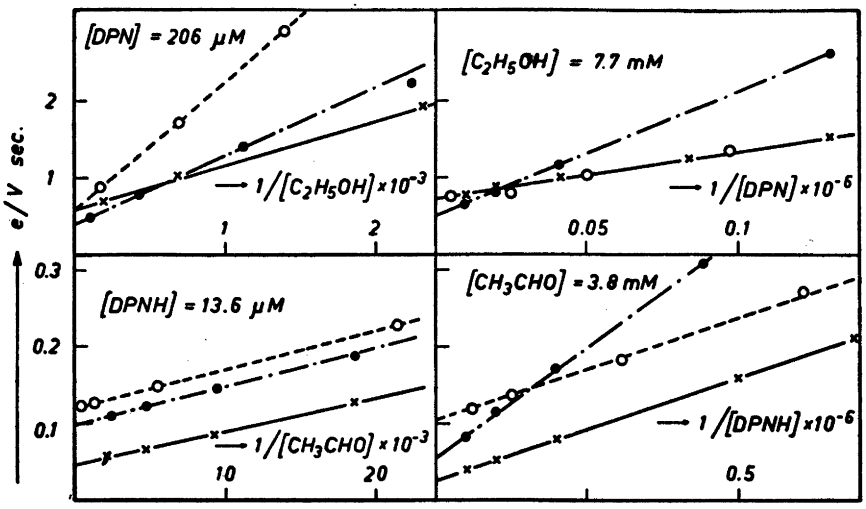


Fig. 3. Lineweaver-Burk plots of three sets of experiments according to Table 1. pH = 7.15, 23° C.

× *Phosphate buffer ionic strength = 0.1*
● » » » » = » + *0.15 M NaCl*
○ » » » » = » + *0.015 M Na formate*

Dissociation velocity constants

A comparison of the new and the old data for k_1 and k_2 reveals that the values between pH 6.8 and 9 agree fairly well, but at pH 10 we now found $k_2 = 2$ and $k_3 = 0.7$, whereas 5 and 4.5 was obtained in the previous work.

Association velocity constants

The present value for k_1 at pH 7.15, 3.7×10^6 $M^{-1} \times sec^{-1}$, agrees remarkably well with the older value 4×10^6 obtained by the independent method of rapid spectrophotometry [2] of the band shift 340—325 mμ in the direct reaction DPNH + ADH → DPNH · ADH.

In the previous work k_5 was computed in an indirect way to be 2×10^6 $M^{-1} \times sec^{-1}$. This value is far too high as compared with the present value at pH 7, 0.3×10^6. The discrepancy is explained by the fact that the old value was based upon an uncertain value for D_{red}, obtained by spectrophotometry of very dilute (~1 μM) solutions of DPNH and ADH (for definition of D_{red}, *cf.* Table 2 and Ref.[1]). Thus contrary to our previous conclusion, k_5 is appreciably lower than k_1, around 12 times at neutral or alkaline reaction, still more in acidic medium.

The new value for k_4 [H+] at pH 7.15, 0.24×10^6 $M^{-1} \times sec^{-1}$, agrees with the old value for k_4 *, 0.22×10^6. This is not surprising, since both values

* In the previous work [2] k_4 was taken as $k_4 = \frac{dc}{dt} \times \frac{1}{[DPNH \cdot ADH][ald]}$; in the present we have preferred to design $k_4 = \frac{dc}{dt} \times \frac{1}{[H^+][DPNH \cdot ADH][ald]}$. The previous k_4 is thus = k_4 [H+] in the present paper.

Table 5. Our kinetical data inserted into three formulas representing reaction sequences.

pH	K_{exp} $\times 10^{-11}$	Two complexes formed. Mech. proposed by Theorell &Chance[2] = Mech. 9 of Alberty [3] $K^* = \frac{k_2 \cdot k_5 \cdot k_6}{k_1 \cdot k_3 \cdot k_4} \times 10^{-11}$	Three or four complexes formed Mech. 7 of Alberty [3] $K = \frac{k_3 \cdot k_5 \cdot k_6}{k_1 \cdot k_2 \cdot k_4} \times 10^{-11}$	Substrate or co-enzyme is oxidized/ reduced by enzyme Mech. 14 of Alberty [3] $K = \frac{k_5 \cdot k_6}{k_1 \cdot k_4} \times 10^{-11}$
5.3	0.86	0.5	230	10.7
6.0	0.86	0.95	640	24.4
7.1	0.86	0.37	197	8.6
8.0	0.86	0.73	114	9.1
9.0	0.86	0.95	6.6	2.5
10.0	0.86	2.6	0.32	0.91

* Alberty's expression is $K = \left(\frac{V_f}{V_r}\right)^2 \frac{K_{Ald} \cdot K_{DPNH}}{K_{Alc} \cdot K_{DPNH}}$, where V_f = max. velocity for the oxidation of alcohol (= k_3) and V_r = max.velocity for reduction of aldehyde (= k_5). When our expressions for Michaelis constants are inserted into the above equation $\left(\frac{V_f}{V_r}\right)^2 \frac{K_{Ald} \cdot K_{DPNH}}{K_{Alc} \cdot K_{DPN}}$ becomes equal to $\frac{k_2 \cdot k_5 \cdot k_6}{k_1 \cdot k_3 \cdot k_4}$. The expressions for Mech. 7 and 14 of Alberty [7] are formed in analogous ways.

were determined from initial velocities with low concentrations of ADH. But it should be pointed out that these values are of the same order of magnitude as the value determined by rapid spectrophotometry of the complex, DPNH · ADH + acetaldehyde (k_4 [H$^+$] $\sim 0.1 \times 10^6$ M$^{-1} \times$ sec^{-1}). Moreover, the last value was certainly too low, since we know from the present work that D_{red} is considerably higher, and [DPNH · ADH] must thus have been lower than calculated in the previous work. The rather satisfactory agreement between the values for k_4 [H$^+$] with acetaldehyde, obtained by two independent methods must be regarded as a strong support of the postulated reaction mechanism.

k_6 was not calculated from the old data [1,2].

The equilibrium constant

K, as calculated from the six initial velocity constants (see Table 4) is sometimes higher, sometimes lower than our previous mean value from equilibrium determinations, 0.86×10^{-11}. It should be pointed out that considerable sources of error exist. For example, it was not possible to make all the four sets of experiments indicated in Table 1 on the same day. For technical reasons the experiments with DPNH and aldehyde had to be made on one day, the experiments with DPN and alcohol on another. As seen from the formula $K = \frac{k_2 k_5 k_6}{k_1 k_3 k_4}$ all the constants in the numerator derive

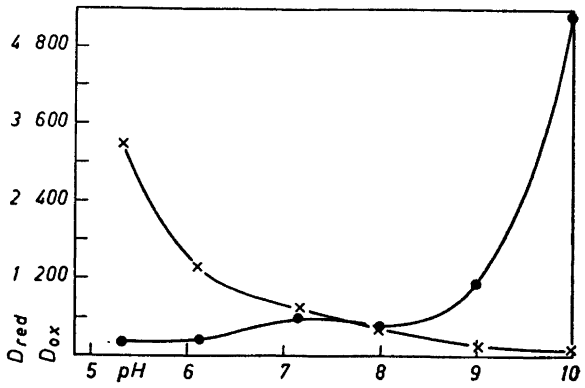


Fig. 4. Variation of D_{ox} and D_{red} with pH.
× D_{ox} μM
● D_{red} »

from the latter sets of experiments, all the constants in the denominator from the former. Errors in the determination of the enzyme concentration, or the calibration of the deflections of the recorder will therefore appear elevated to a potency of three in K (and two in D_{ox}/D_{red}). Therefore we venture that the agreement is as good as could be expected, and adds evidence to our postulated reaction sequence. This is clearly evident when we compare our new values with those calculated from Alberty's [3] main alternative mechanisms, see Table 5.

Alberty [3] found our old data at pH 7 and 8 to agree best with his mechanism 14, at pH 9 and 10 with mechanism 9 (the same mechanism as postulated in this paper). Our present data, on the basis of the accurately determined kinetical constants agree from pH 5.3 to 10 by far best with mechanism 9. It should be noticed how very sensitive the value of K becomes to errors in k_2 and k_3 (= V_f and V_r, respectively) when Alberty's formula for mechanism 9 is used, because it contains (V_f/V_r) [3]. Alberty used our old value $k_2 = 1.1$ at pH 7, whereas our present value at pH 7.15 is $k_2 = 1.6$. Already this discrepancy accounts for threefold error in K.

The dissociation constants of the enzyme-coenzyme compounds

D_{ox} $(= \frac{k_3}{k_5})$ decreases rapidly and continuously with increasing pH, whereas D_{red}, though less markedly, moves the opposite way (see Fig. 4). Possible reasons for this peculiar fact will be discussed later.

As a consequence of this opposite pH-dependence of the dissociation constants, the ratio D_{ox}/D_{red} is very strongly influenced by pH. This was already shown in the equilibrium data obtained in the previous work, from which this ratio was found to be approximately 200, 100, 10 and 3 at pH 7, 8 and 10 respectively. The present data, based upon initial velocity measurements and therefore independent of the equilibrium measurements, confirm the older

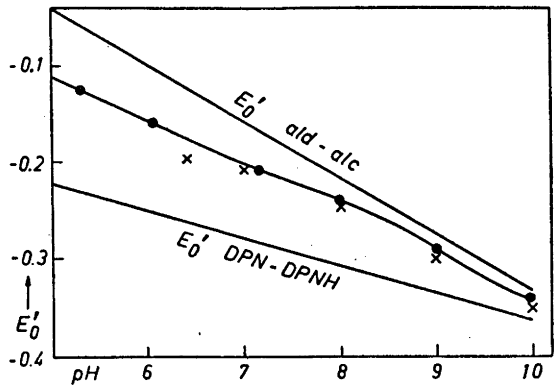


Fig. 5. Oxidation reduction potential of coenzyme-enzyme complex at different pH-values, calculated from D_{ox}/D_{red}.

● *new data*
× *equilibrium data from 1951* [1]

data (from pH 7 to 10). The new values are a little higher (see Table 4), but this is to be expected, because we can now see from our new data for D_{ox} and D_{red} that the excess of ADH was not quite sufficient in the equilibrium experiments of 1951 to make the concentrations of free DPN and free DPNH negligible.

As pointed out before [1] the oxidation-reduction potential of the enzyme-coenzyme complex can be calculated from the formula E_0' (enzyme-coenzyme) $= E_0''$ (coenzyme) $+ \frac{RT}{nF} \ln \frac{D_{ox}}{D_{red}}$. The oxidation-reduction potential of the enzyme-coenzyme compound at different pH-values, using the new values for D_{ox}/D_{red} are plotted in Fig. 5, where the older values are also included for comparison. The new values do not differ very much from the older; and a break in the curve from the slope 0.030 V/pH to 0.060 V/pH occurs, as before, near pH 7.8, indicating a titrable group in DPN · ADH that has not the same pK' in DPNH · ADH.

pH-dependence of the velocity constants

Fig. 6 shows the values for k_2 as a function of the pH. They coincide rather well with a monovalent dissociation curve with p$K' = 6.3$ and varying in height from the hypothetical value 0, at strongly acidic reaction to ~ 1.8 at alkaline reaction. In the presence of excess of both DPN and alcohol, k_2 determines the overall activity. The enzyme system does not show any "pH-optimum" under these experimental conditions but a fairly constant reaction rate in the whole pH-range 7 to 10.

k_3 can be interpreted in terms of two monovalent dissociation curves with pK':s at 6.4 and 7.8 (see Fig. 7). The hypothetical maximum of $k_3 = 60$ is never reached because of the interaction between the two groups. The plot of k_3 *versus* pH has the bell-shape which is so often observed when enzyme

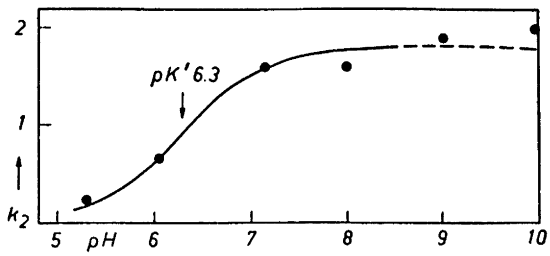


Fig. 6. Variation of k_2 *with pH.*

activity is measured as a function of pH, and which Michaelis attributed to titrable groups of importance for the activity. In our system k_3 is decisive for the overall activity of the enzyme under certain conditions, that is, when both DPNH and aldehyde are in excess. The ADH system under these conditions has its "pH-optimum" at pH 7.1.

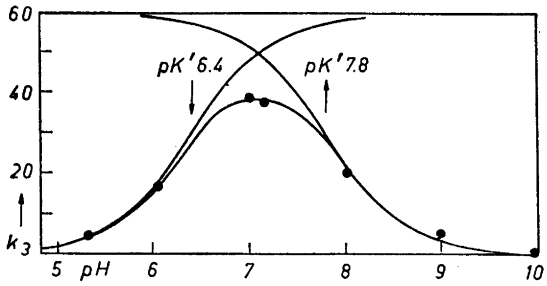


Fig. 7. Variation of k_3 *with pH. The values are close to a difference curve between two monovalent dissociation curves with* pK:s *6.4 and 7.8.*

k_1 and k_5 are both maximal between pH 7 and 8 (see Fig. 8) and both decrease proportionally towards the alkaline side, the pK' being approximately 9 in both cases. It should be noticed, however, that k_1 is here more than 10 times as great as k_5. In the acidic range k_5 begins to decrease at a higher pH (pK' = 6.5) than k_1 (pK' = 5.5), so that at pH 5.3 k_1 is roughly 100 times as

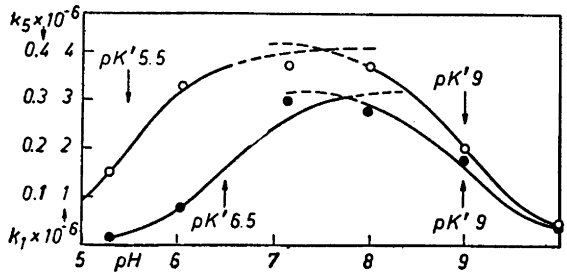


Fig. 8. Variation of k_1 (○) *and* k_5 (●) *with pH.*

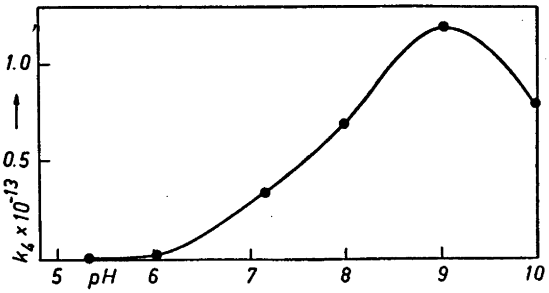


Fig. 9. Variation of k_4 *with pH.*

great as k_5. The "pH optimum" of ADH with excess of acetaldehyde and small amounts of DPNH occurs slightly above pH 7. The same should be the case for the reverse reaction: excess of alcohol, small amounts of DPN.

k_4 versus pH has a more complicated shape (see Fig. 9). It increases from very low values at pH 5.3 and 6 towards the alkaline side, where a flat maximum is found near pH 9. The plot cannot be interpreted in terms of a monovalent dissociation curve.

The pH-optimum for ADH with excess of DPNH and small amounts of acetaldehyde is again found at pH 7, because k_4 $[H^+]$ is then rate-limiting. The maximal reaction velocity in the system ADH + DPNH + acetaldehyde is thus always found near pH 7, though it should be noticed that the maximum is *narrow* with [DPNH] = ∞, [ald] small; *broad* with [DPNH] small, [ald] =∞; and intermediate with both [DPNH] and [ald] = ∞.

k_6 varies along a bell-shaped curve with a maximum near pH 8 (this was already evident in our previous paper (Fig. 8, curve III) and the branches fitting fairly well with monovalent dissociation curves of pK 7.5 and 8.8; see Fig. 10.

The effect of anions

A survey of the effect of different anions on the liver-ADH system revealed strong effects of such anions as chloride, bromide, nitrate and sulfate. This

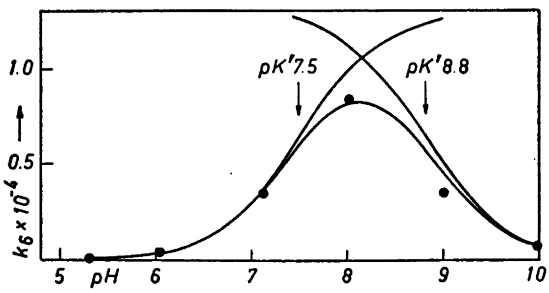


Fig. 10. Variation of k_6 *with pH. The experimental values are close to a difference curve between two monovalent dissociation curves with* pK:s *7.5 and 8.8.*

was expected from the work on the old yellow enzyme. But it was surprising to find that both acetate and formate, which were inactive in the O.Y.E. system, had strong effects on the liver-ADH system. In contrast, versene, which strongly inhibits the reassociation of the old yellow enzyme, did not affect the ADH system. This is shown in Table 6.

Table 6. Influence of some sodium salts, 0.15 M, on the reaction velocity, V/e sec^{-1}, in the ADH—DPN—DPNH system. pH = 7.1, phosphate buffer, ionic strength 0.1, 23.5°.

	ADH [1] = 0.13 μM CH_3CHO = 2 100 » DPNH = 9.6 »	ADH [1] = 0.13 μM C_2H_5OH = 2 300 » DPN = 8 »
Salt	*V/e* sec^{-1}	*V/e* sec^{-1}
Glycine	15	0.32
Phosphate	8.7	0.26
» + versene	8.9	—
Chloride	3.5	0.097
Sulphate	2.3	0.059
Nitrate	1.6	0.013
Bromide	0.13	0.055
Formate	0.17	0
Acetate	—	0.059

In the present investigation we determined the six velocity constants in the presence of 0.15 M sodium chloride and 0.015 M sodium formate at pH 7.15. The results are shown in Fig. 3 and Tables 3 and 4. 0.15 M chloride decreased the rate of association of DPN and DPNH with ADH (k_1 and k_5) to about half, and increased k_2, the dissociation velocity constant of the reduced complex. A similar increase in dissociation velocity by chloride ions was observed in O.Y.E. However, chloride decreased the rate of dissociation of the DPN·ADH complex (strong decrease in k_3) and such an effect was not observed in the O. Y. E. system by any anions. This suggests that other types of linkages may exist between DPN(H) and ADH than between FMN and the apoenzyme of O.Y.E. 0.015 M formate was found to affect only two of the six constants, k_3 and k_6. This means that formate reacts with the DPN·ADH complex, but neither with DPN or ADH before the complex is formed nor with the reduced complex. Formate may enter into the binding site for ethanol in DPN·ADH, since it decreased k_6 competitively. Formate had a similar effect on the yeast alcohol dehydrogenase system.

DISCUSSION

Our postulated reaction mechanism according to eqs. 1—3 has now been found to fit rather well with new experimental data over the whole range of pH from 5.3 to 10. The mechanism apparently does not take into consideration the formation of enzyme-substrate compounds. This may seem surprising

especially in view of the works of Kaplan *et al.*[11] and Vennesland *et al.*[12]. However, as Chance [13] has pointed out, our scheme simply requires that aldehyde-enzyme or alcohol-enzyme complexes break down with rates exceeding the dissociation velocities of DPNH·ADH (k_2) and DPN·ADH (k_3), respectively, and therefore are not revealed as ratelimiting steps. Our constants k_4 and k_6 may thus very well be complicated functions of several intermediate steps without this circumstance interfering with the validity of our results. The accuracy of these results is scarcely high enough to entirely rule out *some* rate-limiting influence of enzyme-substrate compounds; but they can certainly not have large effects on the over-all kinetics of the system. The reaction mechanism for liver-ADH seems to be quite different from yeast ADH [14,15]. Some of our experiments also indicate the same.

The binding sites of ADH for DPN and DPNH

Vallee [16] recently found that yeast ADH contains 4 atoms of zinc per molecule. This interesting finding prompted us (in cooperation with Dr. W. Rutter) to analyze liver ADH for metal content. It was found to contain 2 atoms of Zn per molecule, thus one for each of the two DPN(H) molecules that can be bound to one molecule of liver-ADH. There is as yet no conclusive evidence to show that zinc is essential for the activity of ADH, but it appears likely.

As in the case of the O.Y.E. [5] the shape of the pH dependence curves for the "on" (k_1 and k_5) and "off" (k_2 and k_3) velocity constants may help to reveal the chemical nature of the binding sites. In this connection it is important to remember that changes in k_2 and k_3 are entirely connected with changes in the DPNH·ADH respectively DPN.ADH *complexes*, whereas changes in k_1 or k_5 derive from changes either in the *free* coenzymes, or *free* ADH.

Let us first consider the dissociation constants of the titrable groups in DPN and DPNH. They both contain two primary phosphoryl groups with low pK':s, certainly far below the lowest pH value, 5,3, used in our experiments. The pyridinium ion in DPN is a strong base, but the tertiary nitrogen atom in DPNH is a very weak base with pK' below 4 to 5 [17]. Therefore, within our pH range (5.3 to 10) DPN is carrying a positive charge in the pyridine ring which is absent in DPNH.

The pK' of the primary amino groups of the adenine moiety may not have been accurately determined; however, it is expected to be below 5 (from the fact that pK' for adenine is 4.15) and thus carrying no charge in our pH region. Furthermore, it is known that desamino DPN reacts as rapidly with ADH as does DPN [18]. It is thus likely that the $-NH_2$ group in adenine is unimportant for the coupling of DPN(H) to ADH, although we cannot exclude the possibility that —OH (in desamino —DPN(H)) is bound to the same site in ADH, as is $-NH_2$ (in DPN(H)) and thus is able to replace its function. Summarizing, there appears to be no titrable groups in DPN(H) between pH 5.3 and 9—10. Therefore, we need not to consider the possibility that changes with pH in k_1 or k_5 should depend upon the coenzyme. The extra plus charge in DPN^+ compared with DPNH is, however, important.

The titrable groups in the ADH are probably of the same general nature as in any protein, but in addition we have to take into account the presence of

the zinc atoms that are likely to carry a higher positive charge in acidic than in alkaline solutions.

1. As seen from Fig. 8, both k_1 and k_5 have the same general shape as in the case of the "old yellow enzyme", the "on" velocities being high around neutral reaction, decreasing towards both sides on the pH-scale. As in the case of O.Y.E. the decrease towards the alkaline side might depend upon discharge of primary amino groups, which serve in the binding of the pyrophosphate group in DPN(H). The decrease in k_1 and k_5 towards the acidic side cannot have the same cause as assumed in the O.Y.E., where the secondary phosphate group of FMN is titrated around pH 6, since DPN(H) has no such groups. We have so far no explanation to give.

2. The fact that k_1 is 10—100 times higher than k_5 could be connected with the fact that DPNH has two negative charges, DPN a net sum of only one. Repulsion between the positively charged pyridinium ring in DPN and Zn^{++} could cause the difference between k_1 and k_5. This explanation fits with the fact that the positive charge of Zn is higher in acid solution, where the difference between k_1 and k_5 is greater than in neutral or alkaline solution.

3. The pH-dependence of k_3 is most interesting. DPN · ADH shows the highest dissociation velocity around pH 7, decreasing towards both sides of the pH-scale. This is opposite to what was observed in the O.Y.E.[5]. It may be pointed out that a high dissociation velocity at physiological pH is favorable for the function of an enzyme system with "mobile" coenzyme (ADH), whereas the opposite is true for an enzyme with immobile coenzyme (O.Y.E.).

We think, as before [1], that the increased stability of the DPN . ADH complex towards the alkaline side depends upon an acidic group with $pK' = 7.8$, perhaps a sulfhydryl group, in the vicinity of the pyridinium ring. This effect would be inoperable in the DPNH · ADH complex, where the pyridine ring is uncharged; in accordance herewith k_2 remains constant in the alkaline range. The parallel decrease of k_2 and k_3 towards the acidic range could be caused by imidazole groups near the pyrophosphate groups of DPN(H) taking on positive charge in acid solution ($pK' = 6.4$).

4. The possibility should be pointed out that zinc, which is firmly bound to the ADH, probably by several of its possible six covalent bonds, could use one or two of the residual ones to bind and activate the substrates.

Anion effects

The effects of chloride and formate have so far been studied only at pH 7.15. Therefore a detailed discussion on the conclusions to be drawn from their influence on the ADH-system has to await further data. However, it is seen from Table 3 and Fig. 3 that both formate and chloride decrease k_3, which means a stabilization of the DPN · ADH complex; and both cause a decrease in k_6, which means that they inhibit the reaction of DPN · ADH with alcohol. In addition, chloride decreases k_1 and k_5, and increases k_2. As seen from Fig. 3 this leads to the remarkable effect that chloride stimulates the reaction velocity in a system with high concentrations of both DPN and alcohol, but inhibits when either of them is low.

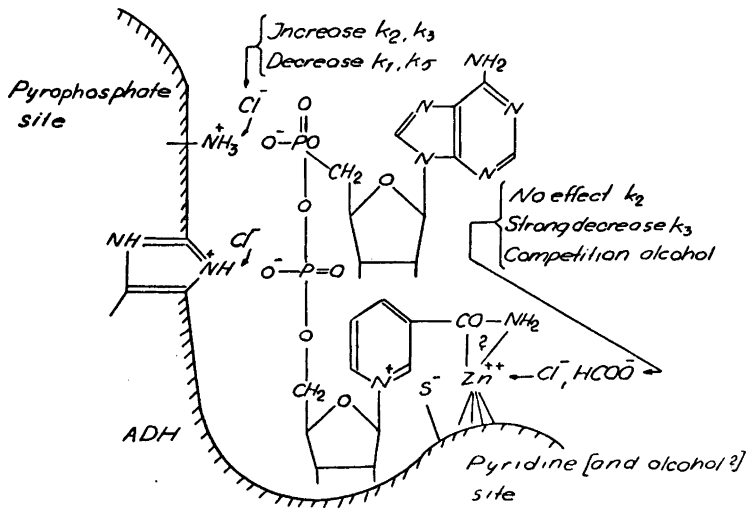
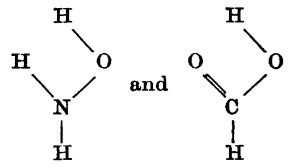


Fig. 11. Tentative scheme for anion effects on the DPN · ADH complex.

The following is a working hypothesis for the explanation of the anion effects: The anions interact with two different binding sites for DPN, the pyridine site and the pyrophosphate site. From our experience with O.Y.E.[5] we would expect chloride to increase k_2 and k_3 and perhaps decrease k_1 and k_5 at the pyrophosphate site (depending upon pH). This is what happened except that k_3 was instead decreased by chloride. Formate likewise decreased k_3, but had no effect on k_1, k_2 or k_5. It appears reasonable to assume that the effect on k_3 of both formate and chloride is at the pyridine site, where the different behaviour of DPN and DPNH is explained by the positive charge which is present in DPN and not in DPNH. Anions could increase the stability of this binding by attaching themselves to a positive group, for example zinc, thereby releasing its repellant effect on the pyridinium ring. This effect would be absent in DPNH · ADH. If the positive group (Zn ?) served as binding site for alcohol, the competition between anions and alcohol could be understandable. The effects of chloride and formate as explained above, are pictured in Fig. 11. It should be unnecessary to emphasize that the illustration is highly speculative.

The fact that formate acts at least 10 times stronger than chloride on k_3 and k_6 indicates that formate fits especially well into the pyridine binding site. This could perhaps be related to the effect of hydroxylamine, studied by Kaplan and his associates [11]. Hydroxylamine, just as formate, has a strongly inhibitory effect on the oxidation of ethanol by DPN · ADH; they both compete with the alcohol; and they both increase the stability of the DPN · ADH linkage. The similar effects are understandable in view of the resemblance of the formic acid and the hydroxylamine molecules



Kaplan *et al.*[11] found that the DPN · NHOH · ADH complex has an absorption band at 300 mμ of about the same height as the DPNH-band at 340 mμ. We therefore investigated whether a similar band could be traced in solutions of DPN and ADH (equimolar amounts) with increasing amounts of formate, at pH 7. No extra bands were found in the ultraviolet, not even with high concentrations of formate. Hydroxylamine and formate are thus different in this respect.

The opposite effects of chloride on k_2 and k_3, and the decreasing effect of formate on k_3 without change in k_2, lead to a change in D_{ox}/D_{red}, as seen from Table 4. This means that the oxidation-reduction potential of the enzyme complex at pH 7.15 will be lowered by 15 mV by 0.15 M chloride and by 17 mV by 0.015 M formate.

The anion effects on the liver-ADH system are in some cases amazingly strong, as seen from Table 6. The results show how extremely carefully the ionic conditions and not only pH have to be considered when studying enzyme-coenzyme reactions.

The effect of anions on the redox potential of the ADH system led us to consider the idea that a salt-sensitive system consisting of an oxido-reducible part reversibly attached to a "carrier" might be operating in certain nerve receptors, transforming a change in ion concentration to a potential change and an electrical discharge. Some recent work by Zotterman *et al.*[18] give some support to this hypothesis. They found particular "water taste" receptors in the cat tongue, that responded with electrical discharges in the corresponding "water fibres" when water, or acetate, or formate solutions were rinsed over the tongue. Salts of strong acids (NaBr, NaI, $NaNO_3$, NaCl, KCl and $CaCl_2$) all abolished this response. It should be recalled that acetate and formate were without effect on the dissociation of the old yellow enzyme, whereas the anions of strong acids all caused increased dissociation, possibly changing the redox potential as in the case of ADH.

Finally, the strong effect of substances like cyanide, hydroxylamine, pyruvoxim and formate on the reaction velocity in the ADH-system is perhaps of considerable interest from a wider point of view. There is no reason to believe that any of these substances, with the possible exception of formate, are present in detectable concentrations in the living cells. But since these chemically very different substances can all react with the pyridine ring, we shall have to look for such effects with substances known to occur physiologically in the cells. This may lead to the discovery of mechanisms for the coordination and regulation of the different enzymatic activities in the cells.

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