

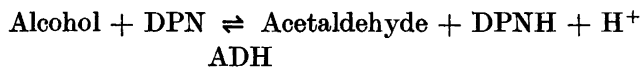
Studies on Liver Alcohol Dehydrogenase

I. Equilibria and Initial Reaction Velocities

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When reduced diphosphopyridine nucleotide (DPNH) forms a compound with horse liver alcohol dehydrogenase (ADH) the absorption maximum of DPNH shifts from 340 $m\mu$ to 325 $m\mu$ ¹. This observation opened up a possibility to carry out a more complete study on the stoichiometry, equilibria and kinetics in an enzyme-DPN system than had before been feasible. The reversible reaction studied in these two papers is:



The equilibrium constant,

$$K = \frac{[\text{DPNH}] [\text{Acetaldehyde}] [\text{H}^+]}{[\text{DPN}] [\text{Ethanol}]}$$

earlier determined by Negelein and Wulff² at pH 7.9, was recently redetermined at different pH values by Racker³, using small concentrations of yeast alcohol dehydrogenase. He found about the same value as Negelein and Wulff at pH 7.9, $K = 1.15 \times 10^{-11}$, and furthermore found K to be independent of pH within the investigated range, in agreement with the above formula.

The kinetic properties of yeast alcohol dehydrogenase were studied to some extent by Negelein and Wulff². They assumed that alcohol and acetaldehyde, as well as DPN and DPNH, combined reversibly with the protein, and found the "dissociation" constant to be 2.4×10^{-2} for ethanol-enzyme and 1.1×10^{-4} for acetaldehyde-enzyme; 9×10^{-5} for DPN-enzyme and 3×10^{-5} for DPNH-enzyme. However, in the theory developed by Negelein and Wulff no distinction was made between Michaelis constants and true dissociation constants.

As all the experiments of Negelein and Wulff were carried out at pH 7.9 the effect of variation in pH upon the kinetics was not investigated. Since hydrogen ions take part in the reaction it seemed to us to be of great interest to investigate the equilibria and reaction velocities at varied pH values.

There was another problem in this connection that attracted our attention. Practically all kinetic work on pyridine nucleotide enzyme systems has hitherto been carried out with very small concentrations of the enzymes, compared with the DPN or TPN concentrations. A rough calculation, however, shows that this ratio may be entirely unphysiological. Since the liver contains about 1 g of enzyme (ADH) per kg wet weight ⁴ and the molecular weight (see below) is 73 000 the liver alcohol dehydrogenase concentration is of the order of 10^{-5} M. If the ADH is located to parts of the liver cells, its concentration must be still higher there. The total concentration of DPN is higher ⁵ but we have to bear in mind that the DPN has to serve as prosthetic group for many other enzymes, so that the concentration of free DPN and DPNH is probably very low in the living cells. A difference between the dissociation constants of DPN-ADH and DPNH-ADH will cause the equilibrium constant K to be dependent on the ADH concentration. It might thus turn out to be a question of enzyme concentration whether the reduction of acetaldehyde or the oxidation of alcohol is preferred.

The substrates react only with the undissociated coenzyme-enzyme compounds. The redox potentials of these are a function of the ratio between the dissociation constant for the DPN-ADH complex (D_{ox}) and the DPNH-ADH complex (D_{red}). This follows from the simple fact that the enzyme-bound part of the coenzymes must be in potential equilibrium with the free coenzymes. The ratios between the concentrations of oxidized and reduced coenzyme in free and bound form, however, will evidently be a function of the dissociation constants of the complexes. Clark and his associates ⁶ have studied this effect in metalloporphyrin complexes with bases and derived the equations for different cases, but insufficient attention has still been paid to its implications in the pyridine- or flavine nucleotide enzyme systems. In the DPN-DPNH systems the following formula is found to obtain (at 30°):

$$E'_0 \text{ (complex)} = E''_0 \text{ (free DPN-DPNH)} + 0.030 \log \frac{D_{ox}}{D_{red}}$$

The assumption is made that the radical formation constant is low. If the ratio $\frac{D_{ox}}{D_{red}}$ is > 1 the redox potential in the case of ADH will be raised from the low level for the free DPN, DPNH equilibrium (-0.282 V at pH 7 and 30° /Borsook ⁷) to a level nearer the normal potential of alcohol, acetaldehyde

(-0.163 V at pH 7, 25° /Kalckar⁸). The reaction velocity in the system would probably be favoured by the diminished potential difference, and the equilibrium shifted so that the oxidation of ethanol would be favoured.

We therefore undertook to study the equilibria between the reaction partners at pH 7, 8, 9 and 10 by the aid of spectrophotometry in the region 310—350 $m\mu$. When the ADH concentration was increased to the "physiological" level around 1 mg per ml very marked effects on the equilibrium constant appeared.

Calculating the stoichiometry of the reactions required the molecular weight of the pure ADH to be known. Dr. K. O. Pedersen, Institute of Physical Chemistry, Upsala, kindly made this determination by ultracentrifugation and diffusion.

Further it was essential to determine the number of coenzyme molecules bound to one molecule of ADH. This was accomplished by titrating a certain amount of ADH with increasing amounts of DPNH, spectrophotometric readings being made at 350 and 310 $m\mu$. Such experiments were carried out at pH 7.0 and 10.0. Further data were obtained from the equilibrium experiments at pH 7, 8, 9 and 10. It was found that one molecule of ADH ($M = 73\ 000$) combined with 2 molecules of DPNH at pH 7—9; at pH 10 the value was nearer to 1 mol DPNH/1 mol ADH.

The kinetics of the ADH-reactions were studied in different ways. The Michaelis constants for DPN, ethanol, DPNH and acetaldehyde, were determined spectrophotometrically in Beckman cuvettes from the initial reaction velocities of the appearance respectively disappearance of the 340 $m\mu$ band.

Most of the experiments on reaction velocities reported in this paper were carried out in Stockholm, but were completed with another set of determinations in Philadelphia. The results of the experiments with rapid spectrophotometry and the theory of reaction velocities in the ADH-system will be given in part II by H. T. and Britton Chance.

EXPERIMENTAL

Molecular weight. The sedimentation constant was found in two runs to be $s_{20} = 4.86$ and 4.90 Svedberg units, the diffusion constant, D_{20} , was found = 6.5×10^{-7} cm^2/sec by the "normal" method, 7.3×10^{-7} by the "moment" method. We determined the partial specific volume to 0.751. From these values $M = 73\ 000$.

It may be mentioned that a sample of yeast alcohol dehydrogenase, crystallized according to Negelein and Wulff² gave $s_{20} = 7.61$ which indicates its molecular weight to be about twice that of liver ADH.

The enzyme used in these experiments was prepared according to an earlier description ^{4,9}. The concentration of the protein was measured from its light absorption at 280 m μ , 1 mg of protein per ml in a 1 cm cell giving an extinction of 0.455 ⁴.

The activity of the enzyme was followed with a Beckman spectrophotometer by measuring the density of the band of DPNH at 340 m μ . The extinction value $\log I_0/I$, 340 m μ , $d = 1$ cm = 0.938 for a solution containing 100 μ g per ml of DPNH was used ¹⁰.

This value can be recalculated to $\epsilon = 6.25$ cm⁻¹ \times mM⁻¹. The activity determination has not before been given in detail. 3 ml solution in a Beckman cuvette is made up to contain: glycine-NaOH-buffer m/10, pH 9.6; 0.75 mg DPN; semicarbazide m/100; 0.05 ml ethanol. The extinction at 340 m μ is determined. A suitable quantity of enzyme, containing 15–20 μ g of pure ADH in a small volume, is placed on a stirring rod and mixed into the cuvette at $t = 0$. The extinction at 340 m μ is read after 3 minutes. Our best preparations have given a density increase of 0.036 in 3 minutes per μ g ADH at room temperature ($\sim 20^\circ$). This gives the maximal turnover number = 140 mol DPN/mol ADH \times min.

This value is considerably lower than the value 220 given by Bonnichsen and Wassén ⁹. Their value, however, was calculated on the basis of a too high extinction coefficient for ADH at 280 (1.0 instead of 0.455), and the assumption of $M = 70\ 000$. The recalculated value, on the basis of $M = 73\ 000$ and $\epsilon_{280} = 0.455$, for their turnover number would be = 110. The difference from our somewhat higher value, 140, is due to the fact that ADH gives beautiful crystals together with some inactive protein that can be removed by repeated recrystallization.

Fig. 1 shows that the reaction velocity with DPNH and acetaldehyde as substrate is strictly proportional to the enzyme concentration. The same applies to the system DPN + ADH + ethanol at higher pH.

The actual concentration of ADH is computed according to the activity test to be

$$[\text{ADH}] = \frac{\mu\text{M DPNH/sec } \mu\text{M}}{2.34}$$

where 2.34 is the turnover number per sec.

The purity of the enzyme can be determined by dividing this value with the concentration of ADH determined from the extinction at 280 m μ :

$$[\text{ADH}] = \frac{\epsilon_{280} \times 10^3}{33.3} \mu\text{M}$$

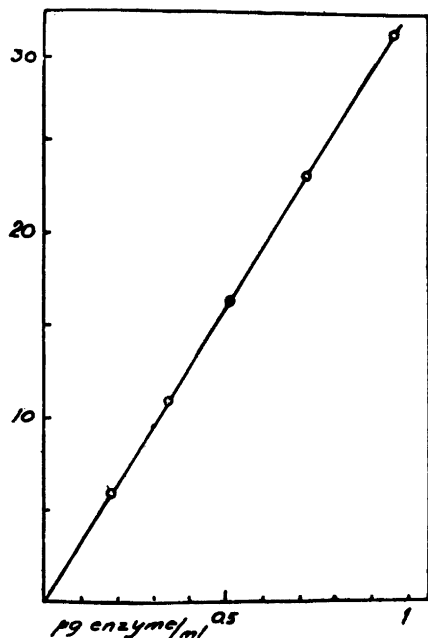


Fig. 1. Initial reaction velocity in μg DPNH per ml per minute with different quantities of ADH of 0.7 degree of purity. $\text{pH} = 6.9$, 0.05 M phosphate buffer. DPNH: $30\ \mu\text{g/ml}$. Acetaldehyde $3 \times 10^{-3}\text{ M}$.

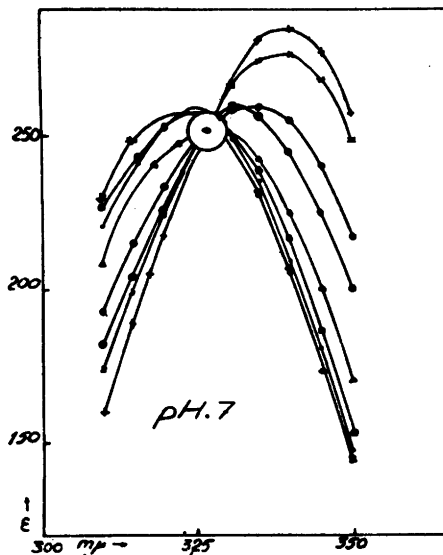


Fig. 2. Light absorption of DPNH with varied concentrations of ADH.

	$[\text{DPNH}] = 0.449 \times 10^{-4}\text{ M}$
\times	$[\text{ADH}] = 0.395$ »
$*$	$= 0.295$ »
\bullet	$= 0.197$ »
\circ	$= 0.148$ »
Δ	$= 0.099$ »
\diamond	$= 0.049$ »
\cdot	$= 0.025$ »
$+$	$[\text{ADH}]$ extrapol. to 0

where ϵ_{280} is the optical density (cm^{-1}) at $280\text{ m}\mu$ and 33.3 is the millimolar extinction coefficient of pure ADH. The optical density at $280\text{ m}\mu$ would include protein impurities.

The DPN was prepared according to LePage¹¹; the purity has varied from 20 to 65%. DPN prepared by the method of Neilands¹², 60 to 85% pure, has also been used; we found no difference in the results with 20 or 80% pure DPN. The measurements of the DPN concentration and the preparation of DPNH were both made with the enzymatic method¹³.

A number of experiments on the equilibria at varied pH and ADH concentration were carried out in 1 cm Beckman cuvettes in a thermostat at 20° . 0.05 m phosphate, or pyrophosphate, or glycine buffers of pH 7, 8, 9 or 10 were mixed with ADH that had been dialyzed against the same buffer, and suitable amounts of dilute ethanol added to make the volume = 3 ml. The

light absorption was measured at every 5 $m\mu$ in the region 310—350 $m\mu$. At $t = 0$, 0.3 ml of DPN solution was added and readings taken at the shortest possible intervals.

The equilibrium was established instantaneously with high concentrations of ADH and then a further slow reduction of DPN followed. In such cases we extrapolated our reading to $t = 0$. When the ADH concentration was low we had to wait until the equilibrium was established. In these cases no further reduction occurred; it was thus very probably caused by some small amount of reducing material in the ADH.

After having made enough equilibrium readings we added more ethanol (0.1 ml, diluted 1 : 10) and semicarbazide to a final concentration of 0.01 M . Full reduction of the remaining DPN was obtained in a few seconds with the high, in 30—60 minutes with the lowest ADH concentrations. The light absorption in the 310—350 $m\mu$ region was read and corrected for the absorption determined before adding DPN. The dilution factors were taken into account.

Fig. 2 shows the extinction values ($\times 10^3$) obtained for the DPNH with increasing amounts of ADH at pH 7. It is seen how the absorption band 340 $m\mu$ of the free DPNH gradually moves towards 325 $m\mu$, and decreases a little. All the curves intersect at 328 $m\mu$; this is thus an isosbestic point for free and ADH-bound DPNH. The millimolar extinction coefficients for bound and free DPNH from $\lambda = 310$ to $\lambda = 350$ $m\mu$ are given in Table 1. The greatest difference in extinction occurs at 350 $m\mu$ ($2.4 \text{ cm}^{-1} \times \text{mM}^{-1}$), whereas the difference at 310 $m\mu$ is somewhat smaller ($1.5 \text{ cm}^{-1} \times \text{mM}^{-1}$). The values in Table I are plotted in Fig. 3. (corr. for the light absorption of ADH!).

The results of the equilibrium experiments at pH 7, 8, 9 and 10 are given in the Tables 2-5. The extinction coefficients are not included in the tables; instead the DPNH extinction coefficients at 310 and 350 $m\mu$ after full reduction are plotted against the molarity of the ADH in Fig. 4.

Table 1. Millimolar extinction coefficients for free, respectively ADH-bound DPNH from 310 to 350 $m\mu$.

$\lambda, m\mu$	310	315	320	325	328	330	335	340	345	350
Free DPNH	3.6	4.1	4.8	5.3	5.65	5.8	6.1	6.25	6.1	5.7
Bound DPNH	5.1	5.5	5.7	5.8	5.65	5.6	5.3	4.7	4.1	3.3

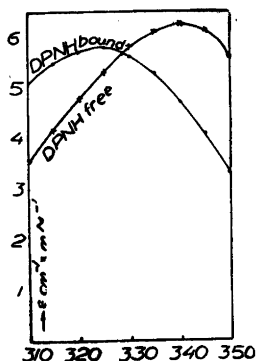
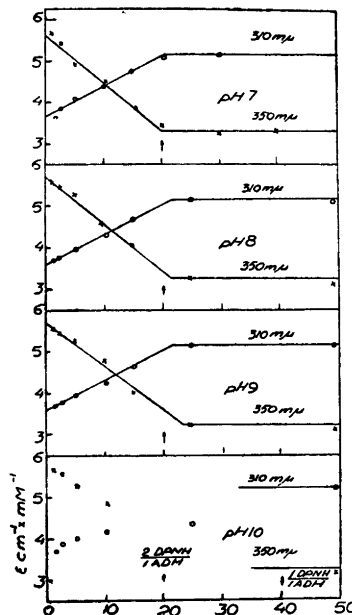


Fig. 3. Millimolar extinction of free and ADH-bound DPNH.

Fig. 4. The extinction coefficients of DPNH calculated from the equilibrium experiments in Tables 2-4. The abscissa gives the micromolarity of the added ADH ($M = 73000$) per $40 \mu\text{M}$ DPNH.



The Fig. 4 clearly shows that two molecules of DPNH are bound to each molecule of ADH at pH 7 and 8 and possibly somewhat less at pH 9. The values at pH 10 were more uncertain, but not far from 1 mol DPNH per mol ADH. It is further evident that both molecules of DPNH are so tightly bound to the ADH at pH 7-9 that no dissociation constants can be calculated at concentrations of the components. These determinations therefore had to be carried out at much higher dilutions with a more sensitive instrumental arrangement (See part II).

Some direct spectrophotometric titrations of a certain amount of ADH with increasing amounts of DPNH were carried out at 310 and 350 $m\mu$ as well in Stockholm as in Philadelphia. These experiments confirmed that 2 DPNH were bound per 1 ADH at pH 7. The values obtained at pH 10 seem to indicate that only 1 DPNH is bound per 1 ADH at this pH, and that the complex is more dissociated than at pH 7-9. A couple of these experiments are plotted in the Figures 5 and 6.

It is seen in the Tables 2-5 that the *equilibrium constant* K is increased with increasing ADH concentration to a new level in the presence of ADH in excess, around 200 times its initial value at pH 7. With increasing pH this effect is diminished, so that at pH 10 very little, if any, is left, see Fig. 7. The slight increase in K with the highest ADH amounts at pH 10 may as well

Table 2. *Equilibria at pH = 7.0, 20°.*

[ADH] × 10 ⁴	[C ₂ H ₅ OH] × 10 ⁴	[DPN] _{Tot} × 10 ⁴	% Red	K × 10 ¹¹	log K + 11	log [ADH] + 7
0.003	10 400	0.40	79.6	1.20	0.08	0.48
0.003	1 040	0.40	40.0	1.02	0.01	0.48
0.012	1 560	0.45	48.2	1.29	0.11	1.08
0.025	1 560	0.45	50.4	1.47	0.17	1.39
0.050	780	0.45	46.8	2.2	0.35	1.70
0.099	260	0.45	46.4	6.7	0.83	2.00
0.099	26.8	0.45	20.4	13	1.11	2.00
0.148	104	0.45	51.2	23	1.36	2.17
0.197	31.2	0.45	40.0	39	1.59	2.30
0.197	5.4	0.34	26.5	80	1.90	2.30
0.296	20.8	0.45	48.2	97	1.99	2.47
0.395	10.4	0.45	46.8	181	2.26	2.60
0.395	5.4	0.40	32.5	146	2.16	2.60
0.790	5.4	0.35	35.5	192	0.28	2.90

Table 3. *Equilibria at pH 8.0, 20°.*

[ADH] × 10 ⁴	[C ₂ H ₅ OH] × 10 ⁴	[DPN] _{Tot} × 10 ⁴	% Red	K × 10 ¹¹	log K + 11	log [ADH] + 7
0.003	1040	0.40	71.4	0.69	- 0.16	0.49
0.003	104	»	35.1	0.73	- 0.14	0.49
0.012	520	»	65.5	0.96	- 0.02	1.09
0.025	520	»	66.3	1.00	± 0	1.39
0.050	156	»	53.5	1.60	0.20	1.70
0.099	26	»	33.0	2.50	0.40	2.00
0.148	10.4	»	40.4	11	1.04	2.17
0.247	5.2	»	50.4	41	1.61	2.40
0.493	5.2	»	64.9	96	1.98	2.70

Table 4. *Equilibria at pH 9.0, 20°.*

[ADH] × 10 ⁴	[C ₂ H ₅ OH] × 10 ⁴	[DPN] _{Tot} × 10 ⁴	% Red	K × 10 ¹¹	log K + 11	log [ADH] + 7
0.003	104	0.40	78.0	1.08	+ 0.03	0.49
0.003	10.4	»	38.8	1.03	+ 0.01	0.49
0.012	31.2	»	58.4	1.06	+ 0.03	1.09
0.050	31.2	»	56.9	0.98	- 0.01	1.70
0.099	10.4	»	40.0	1.05	+ 0.02	2.00
0.148	5.2	»	45.8	3.1	0.49	2.17
0.247	3.6	»	49.5	5.0	0.70	2.40
0.493	2.6	»	54.9	10.0	1.00	2.70

Table 5. Equilibria at pH 10.0, 20°.

[ADH] × 10 ⁴	[C ₂ H ₅ OH] × 10 ⁴	[DPN _{Tot}] × 10 ⁴	% Red	K × 10 ¹¹	log K + 11	log [ADH] + 7
0.003	10.4	0.4	63	0.45	- 0.35	0.49
0.003	1.04	»	41	1.35	+ 0.13	0.49
0.012	3.12	»	50.4	0.70	- 0.15	1.09
0.025	3.12	»	48.5	0.63	- 0.20	1.39
0.050	2.08	»	46.0	0.81	- 0.09	1.70
0.099	2.08	»	47.6	0.92	- 0.04	2.00
0.247	2.08	»	54.5	1.4	0.15	2.40
0.493	2.08	»	62.2	2.3	0.36	2.70

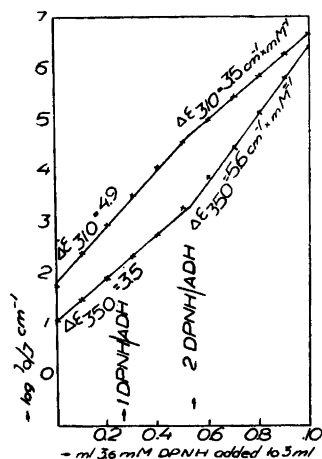


Fig. 5. Spectrophotometric titration of ADH with increasing amounts of DPNH, pH = 7.

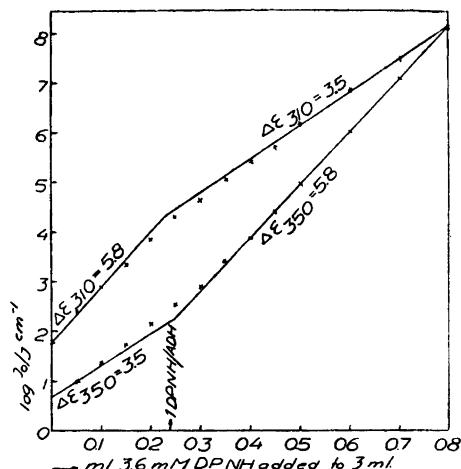


Fig. 6. Spectrophotometric titration of ADH with increasing amounts of DPNH, pH = 10.

be caused by traces of ethanol in the ADH-solution; a few micrograms would be enough to cause this effect at pH 10, but would have no significance at the lower pH-values.

The equilibrium constant, *K*, at low ADH-concentrations (0.004 × 10⁻⁴ M) was found to be independent of pH and = 0.86 × 10⁻¹¹ on an average. This value agrees reasonably well with that found by Racker³, 1.15 × 10⁻¹¹. He used yeast ADH; the difference between the two values may be a temperature effect, since Racker does not give any temperature data. It should be pointed out that *K* is independent of the ratio [DPNH] : [DPN] at low and high [ADH].

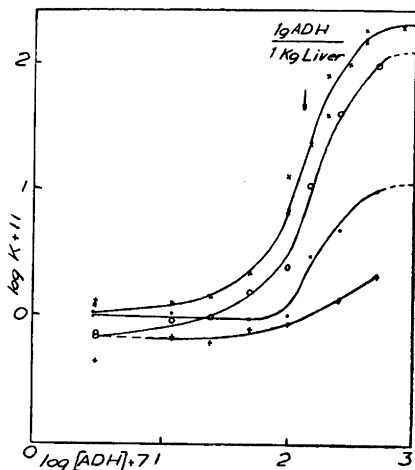


Fig. 7. Equilibrium constant, K , at varied $[ADH]$ ($M = 73000$) and pH . $\log K + 11$ plotted against $\log [ADH] + 7$.
 \times $pH = 7$
 \circ $pH = 8$
 \cdot $pH = 9$
 $+$ $pH = 10$

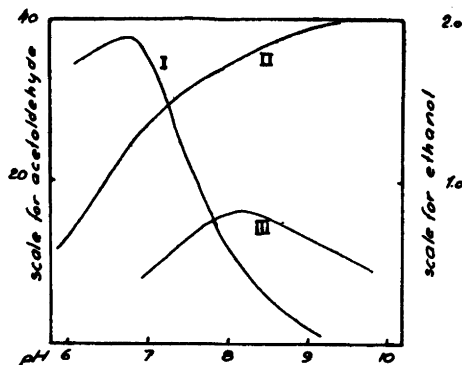


Fig. 8. Initial reaction velocity at different pH values. Buffers = $0.05 M$ phosphate and pyrophosphate. $0.5-1 \mu g/ml$ of ADH and $50 \mu g/ml$ of $DPNH$ were used in the experiments with acetaldehyde; $4-8 \mu g/ml$ of ADH and $180 \mu g/ml$ of DPN with ethanol.

$$v = \frac{c_0 - c_t}{t \times e} \cdot \text{min}^{-1}$$

$$I = \text{Acetaldehyde } 10^{-3} M$$

$$II = \text{Ethanol } 2 \times 10^{-2} M$$

$$III = \text{ » } 5 \times 10^{-4} M$$

but not at intermediate $[ADH]$. This is the reason why a considerable dispersion of the K values occurs in this region. Two determinations of K at pH 6.4, with excess of ADH , gave the values 128 and 134×10^{-11} .

If Borsook's (7) value for the redox potential of the DPN - $DPNH$ system, $-0.282 V$, at 30° , pH 7, is recalculated to 20° , we obtain $-0.275 V$. Kalekar⁸ calculated the potential of acetaldehyde-ethanol from Negeleins and Wulffs data; he obtained the value $E'_0 = -0.163 V$, at 25° . This corresponds to $-0.156 V$ at 20° . Our value of $K = 0.86 \times 10^{-11}$ at pH 7, and 20° would give the following value for the redox potential of acetaldehyde-ethanol:

$$E'_0 (20^\circ C, pH 7) = -0.275 + 0.0291 \log \frac{1}{0.86 \times 10^{-4}} = -0.156 V. \text{ This}$$

is in exact agreement with Negeleins and Wulffs experimental data and Kalekars calculation.

The redox potentials for the ADH -bound coenzymes at pH 6.4—10 were calculated from the maximum values of the equilibrium constants in Tables 2—5, obtained with excess of ADH . The reasonable assumption was made that the slope $0.029 V/pH$ for the free DPN and $DPNH$, and the slope $0.058 V/pH$ for acetaldehyde and ethanol obtains in this pH range. The results are given in Table 6.

Table 6. Values calculated from the equilibrium constants for the redox potentials of the ADH-bound DPN-*DPNH* system.

pH	E'_0 , DPN, <i>DPNH</i> , free	E'_0 , DPN, <i>DPNH</i> bound	E'_0 , ethanol, acetaldehyde
6.4	- 0.258	- 0.196	- 0.121
7.0	- 0.275	- 0.208	- 0.156
8.0	- 0.304	- 0.244	- 0.214
9.0	- 0.333	- 0.302	- 0.272
10.0	- 0.361	- 0.351	- 0.330

It is interesting to notice that the E'_0 for the enzyme complex approaches the level of the aldehyde-ethanol potential very considerably so that at pH 8 the difference is only 30 mV. The slope of the calculated E'_0 curve for the complex is partly steeper than for the free coenzymes, 0.058 V/pH from pH 8 to 9.5. The significance of this observation will be discussed later (p. 1121).

The K -values indicate that the equilibrium states for $[\text{DPN} \cdot \text{ADH}] = [\text{DPNH-ADH}]$ will be ethanol: acetaldehyde = 60 at pH 7, and = 10 at pH 8, instead of respectively $\sim 10\,000$ and 1 000 for the equilibria with small amounts of ADH. The coupling of the coenzymes to the enzyme thus in this case greatly favours the oxidation of ethanol to acetaldehyde — a reaction that would seem rather improbable without this effect. It should be noticed that acetaldehyde, when formed in the liver, is rapidly oxidized by aldehyde oxidase and thus removed from the equilibrium.

The *experiments on initial reaction velocities* were carried out as follows: The Beckman cuvettes were first charged with buffer, coenzyme and substrate. The enzyme was pipetted into a small excavation on the end of a small glass rod and stirred into the solution at $t = 0$. Readings were taken at 15 second intervals. Since the reaction velocity rapidly decreases with time the initial velocity had to be determined by extrapolating to zero time, and all activities of the enzyme referred to initial velocity. As an expression for initial velocity we use $\frac{c_0 - c_t}{t \times e}$ min.⁻¹, $c_0 = \gamma$ DPN/ml at zero time, $c_t = \gamma$ DPN/ml at time t , $e = \gamma$ enzyme/ml.

All the buffers used were made 0.1 % with respect to glycine in order to stabilize the enzyme; our stock solutions of enzyme contained 5–6 mg of

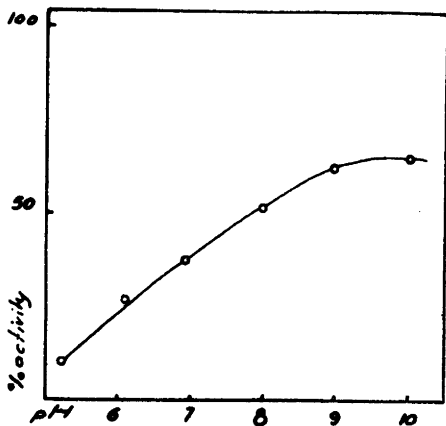


Fig. 9. Inhibition of initial velocity by DPNH. "100 % activity" is = initial velocity with 130 μ g DPN. The inhibition experiments were carried out by mixing 25 μ g of DPNH and 130 μ g of DPN and measuring the initial velocity in phosphate and pyrophosphate buffers.

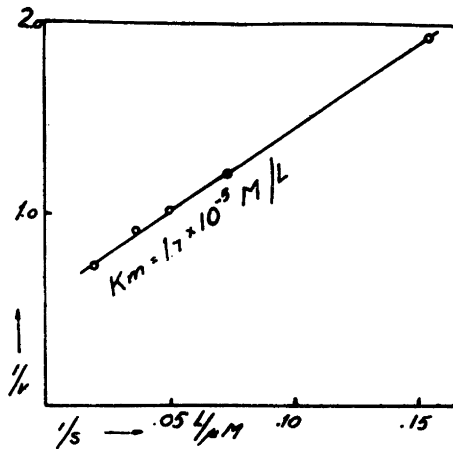


Fig. 11. Michaelis constant K_m for DPN. Alcohol $5 \cdot 10^{-3}$ M, phosphate buffer pH 10.

$$V = \frac{C_0 - C_t}{t \times e} \text{ min}^{-1}, S = [\text{DPN}]$$

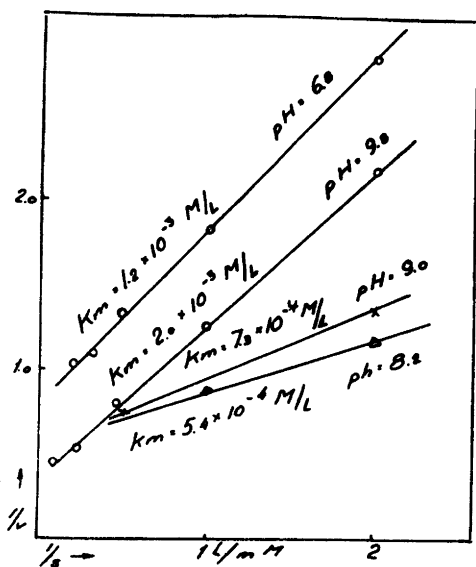


Fig. 10. Michaelis constant, K_m , for alcohol at different pH levels. DPN 180 μ g of enzyme per ml.

$$V = \frac{C_0 - C_t}{t \times e} \text{ min}^{-1}, S = [\text{C}_2\text{H}_5\text{OH}]$$

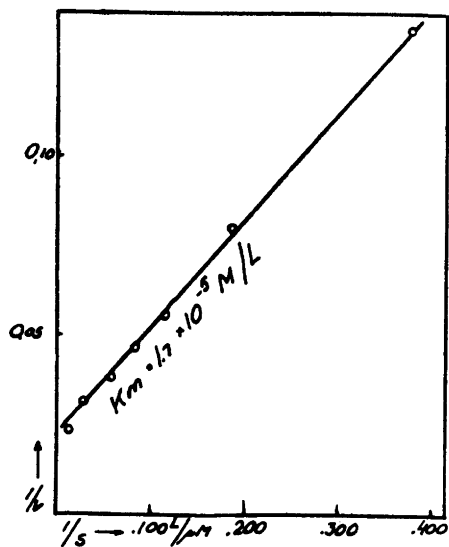


Fig. 12. Michaelis constant K_m for DPNH.

Phosphate buffer pH 10.
Acetaldehyde $5 \cdot 10^{-3}$ M.

$$V = \frac{C_0 - C}{t \times e} \text{ min}^{-1}, S = [\text{DPNH}]$$

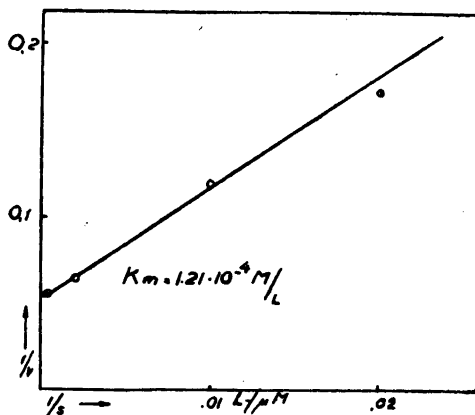


Fig. 13. Michaelis constant for acetaldehyde. Phosphate buffer pH 10; DPNH 6.12 μg per ml. 5 cm cuvettes were used.

$$V = \frac{C_0 - C_t}{t \times e} \text{ min}^{-1}, S = [\text{CH}_3\text{CHO}]$$

enzyme per ml. Dilutions from this stock solution were made for each set of experiments, since solutions containing less than 50 μg per ml retained their full activity for only a few hours. When alcohol was used as substrate the buffers were made 0.01 M with respect to semicarbazide in order to bind the acetaldehyde.

The Fig. 8 shows the results of some experiments on the initial reaction velocities under varied conditions described in the figure legend. It is seen that DPNH and acetaldehyde are brought to rapid reaction by the ADH at pH 6 and 7. At higher pH-values the velocity drops sharply (curve I).

The curves II and III show that the reaction velocity in the system DPN + ethanol + ADH depends upon the pH in a somewhat complicated way. High concentrations of ethanol, far exceeding the Michaelis constant (see below) cause the reaction velocity to increase continuously from pH 6 to between 9 and 10 (curve II), whereas small concentrations (500 μM , curve III) lower than K_m give a velocity maximum around pH 8. The explanation is the difference in limiting factors in the two cases, as will be shown below and in part II.

The initial reaction velocity in the system DPN + ethanol + ADH is decreased in a pH-dependent way by the addition of DPNH (see Fig. 9). The effect is most pronounced at lower pH-values. This experiment shows that DPN and DPNH compete about the same position at the enzyme. It further illustrates the higher affinity to ADH of DPNH compared with DPN.

The Figs. 10—13 illustrate the results of some measurements on the initial reaction velocities under conditions given in the figure legends and plotted in the way suggested by Lineweaver and Burke¹⁴. These experiments were made in Stockholm in the way described above. In the figures, c_0 , c_t and e are given

Table 7.

I	II	III	IV	V	VI
Compound varied in concentration	pH	K_m μM		V_{\max} sec^{-1} corr. (= k_3)	Max. turnover number $\frac{\text{Mol. substrate}}{\text{Mol. ADH}} \text{min}^{-1}$
		Theorell and Bonnichsen	Theorell and Chance		
Ethanol	6.8	1.200		1.1 *	132
	8.2	540		1.5	180
	9.0	730		1.5	180
	9.8	2.000		5.5 *	330
Other alcohols	10	220—410		6.6	400
DPN	7		12		
	8		7		
	9		15		
	10	17	30	4.6	280
Acetaldehyde	7	120		45 ¹	5400
	8		52	17	2040
	9		2500	8	960
	10		1000	4.5	270
DPNH	7	17		39	4700
	7		13		
	8		4		
	9		—		
	10		6		

¹ K_m for DPNH taken as 15 μM .

in μg (γ) per ml. They were completed by some determinations in Philadelphia in the cases where low concentrations and high sensitivity were required (*cf.* part II). The results are summarized in the Table 7.

The values of K_m in column III are those given in the Figs. 10—13. The maximal reaction velocities, V_{\max} , found in these experiments were converted to k_3 (for definition see part II) by using the known molecular weights of DPNH and ADH, and by converting minutes to seconds:

$$k_3 = \frac{1}{663} \times 73\,000 \times \frac{1}{60} V_{\max} = 1.8 V_{\max} \text{sec}^{-1}$$

This value was used at pH 9.8—10, where ADH binds 1 DPNH; but where ADH binds 2 DPNH, thus at pH 6.8—9, the effective molecular weight of ADH is $\frac{73000}{2}$, and

$$k_3 = 0.9 V_{\max} \text{ sec}^{-1}$$

In some cases the concentration of the reaction partner that was kept constant was found to have been insufficient to give maximal reaction velocity at high concentration of the variable partner. The values marked * in column V were then corrected by the aid of the known K_m for the constant partner.

The col. VI gives the maximal turnover numbers of substrate per mol. ADH ($M = 73\ 000$) and minute.

The K_m -values in col. IV are to be regarded as preliminary, especially in the case of acetaldehyde and DPNH, because these may to some extent react directly with one another even without ADH.

It is seen from the Table 7 that the K_m for ethanol passes a minimum around pH 8, and the same is true for DPN and acetaldehyde, whereas the K_m for DPNH shows a minimum at pH 9, too low to be determined with the available technique.

The Michaelis constants, compared with the values for the maximal velocities, readily explain the experimental results shown in Fig. 8. The reaction velocity with acetaldehyde and DPNH (curve I) decreases very rapidly when the pH increases from pH 7 to 10, because the K_m increases at the same time as V_{\max} decreases. The reaction velocity in the DPN + ethanol system increases continuously with increasing pH in the presence of high concentrations of ethanol (II) because V_{\max} increases, and because even the comparatively high K_m at pH 10 does not play any role. At low ethanol concentrations (III) the influence of the minimum in K_m at pH 8 predominates so that a velocity maximum at this pH will result. A pH-value of 9.2—10 is, however, to be preferred for determining ethanol quantitatively¹⁵.

SPECIFICITY

The pure ADH does not react with methanol + DPN to any measurable extent. This is surprising since Røe¹⁶ in clinical cases found ethanol to act as an antidote in methanol poisoning, supposedly by displacing "methyl alcohol from the inner surface of the cells". Zatman¹⁷ in experiments with impure ADH, prepared according to Lutwak-Mann¹⁸, found methanol to be oxidized at one ninth of the rate for ethanol. A competitive inhibition of methanol oxidation by ethanol was observed. The only possible explanation

seems to be that methanol oxidation *in vivo* requires some catalyst besides ADH + DPN.

The higher homologues of ethanol react if they contain the group $\begin{array}{c} | \\ -\text{C}-\text{CH}_2-\text{OH} \\ | \end{array}$. Thus secondary butanol reacts, but not tertiary butanol or isopropanol. Ethylene, glycol, and β -hydroxybutyric acid do not react, neither does acetone (with DPNH + ADH). It is obviously important for the practical use of the ADH-method¹⁵ for determining ethanol in blood samples or organs that methanol, acetone, acetoacetic acid and β -hydroxybutyric acid do not react.

Some experiments on the initial reaction velocity on higher aliphatic alcohols are shown in Fig. 14. The Michaelis constants are lower for these alcohols than for ethanol, but the maximal reaction velocity is practically the same, as required by the theory (*cf.* part II). Lutwak-Mann¹⁸ in experiments with impure horse liver ADH used several alcohols as substrate, and found the highest turnover with ethanol and slightly lower values for propanol. The reaction with amyl alcohol was slow and with methanol very slow.

In 1949, professor R. A. Morton, Liverpool, sent us a sample of crystalline retinene with the suggestion to try whether it could react with DPNH + ADH to give vitamin A. Before our experiments on this problem were carried out, Bliss¹⁹ showed that liver extracts could convert the alcohol vitamin A to the corresponding aldehyde, retinene, and discussed the possibility that the ADH present in his extracts might be responsible for the reaction. This was confirmed in experiments on the reverse reaction with pure ADH + DPNH + retinene, carried out by one of us (R. B.) and R. Hubbard at the Carlsberg Laboratory in Copenhagen. It seems therefore possible that ADH may be present in the retina exerting another important physiological function in the formation mechanism of rhodopsin.

DISCUSSION

It is interesting to compare the normal potential values calculated here with some other oxidoreduction enzymes:

Cytochrome b:	+ 0.04	V
"Old" yellow ferment:	— 0.06	»
Free riboflavin:	— 0.185	»
ADH—DPN(H)	— 0.21	»
DPN(H)	— 0.28	»

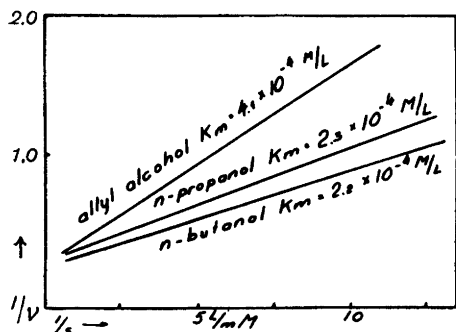


Fig. 14. Michaelis constants for various alcohols. Glycine-NaOH buffer pH 10. DPN 180 μ g per ml. Enzyme 8 μ g per ml.

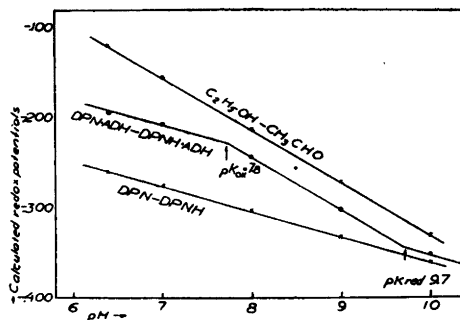


Fig. 15. Redox potentials in the ADH-system. The straight lines for $C_2H_5OH-CH_3CHO$ and DPN-DPNH are calculated according to Borsook (7) and Kalckar (8). The points (o) on the DPN-ADH-DPNH-ADH line are calculated from the equilibrium data at high [ADH].

Unsuitably large potential differences seem thus to be overbridged by nature by the different dissociation constants in the coenzyme-enzyme systems.

In the flavin enzymes it is a well-known fact that absorption maximum at 445—450 $m\mu$ in the free riboflavinmono- or dinucleotides are displaced somewhat towards longer wavelengths when they are coupled to different enzyme proteins, for instance to 465 $m\mu$ in the "old" yellow ferment²⁰. Kuhn and Boulanger²¹ assumed this shift to be due to a linkage between the protein component and the NH (3) group in the riboflavin. In fact we were lead by analogy to look for an analogous shift in the 340 $m\mu$ band in DPNH-ADH.

Since it is a definitely established fact that the 340 $m\mu$ band is caused by the reduced pyridine ring in DPNH, the shift in absorption maximum on coupling with ADH must be caused by some group in the protein being linked in one way or the other to the pyridine. This would give an explanation to the differences in the dissociation constants of the DPN-ADH and the DPNH-ADH compounds.

The calculated redox potentials of the enzyme-coenzyme compound at different pH give additional evidence of the existence of an acid group in the ADH that is linked to the pyridine. As will be seen on Fig. 15 the slope $\frac{dE'_0}{d\text{pH}}$ is = 0.029 from pH 6.38 to pH 7.8, 0.058 up to pH about 9.7, and

probably 0.029 above this value, though further experimental data will be needed to verify this. The observed slopes indicate that an acid group in the DPNH · ADH with pK' 10 is influenced by the oxidation of DPNH · ADH, so that its pK' is changed to 7.8. This is readily understandable since the tertiary, uncharged pyridine-N in DPNH becomes a quaternary $\overset{+}{\text{N}}$ on oxidation. The difference in pK' , two pH-units, is of the expected order of magnitude in such a case. If the DPNH-linked group in the protein is titrated, with $pK' = 10$, so that the bond to the DPNH is broken, one would expect ADH to bind half as much DPNH at pH 10 as at lower pH values, as in fact was found experimentally.

Proteins may contain three kinds of groups with pK -values around 10, amino groups, tyrosine hydroxyls and sulfhydryl groups. G. Wald and R. Hubbard kindly informed us very recently that the ADH-activity was strongly inhibited by *p*-chloromercuribenzoic acid, and that this inhibition was reversed by glutathione. We therefore measured the extinction coefficient of the DPNH—ADH complex at pH 7 before and after the addition of *p*-chloromercuribenzoic acid (0.0003 M). *The maximum at 325 m μ was immediately shifted to 340 m μ upon the addition of the inhibitor.* Monoiodoacetic acid, that according to Lutwak-Mann¹⁸ does not inhibit the ADH activity, had no influence on the position of the band, nor had chloroacetophenone.

These observations seem to demonstrate that 1) *a sulfhydryl group in the ADH is linked to the dihydropyridine in DPNH*, 2) that this linkage causes the band shift from 340 to 325 m μ , and 3) that this linkage is essential for the enzymatic activity of the DPNH—ADH complex. ADH gives no color in the nitroprusside test. The sulfhydryl groups if present are thus masked in some way ("b-groups", Hellerman *et al.*²²) so that they do not react with this reagent nor with monoiodoacetic acid or chloroacetophenone.

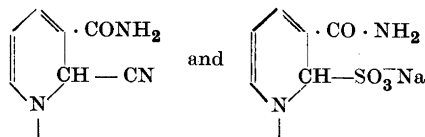
The activity of the alcohol dehydrogenase from yeast is according to Lutwak-Mann¹⁸ inhibited by monoiodoacetic acid. It is therefore possible that this ADH contains unmasked sulfhydryl groups coupling to DPNH in an analogous way as in liver-ADH. We hope to be able to report shortly if the 340 m μ band of DPNH is displaced by the complex formation with yeast ADH.

As far as we know the possibility of a direct interaction of enzyme SH-groups with coferments like DPN and TPN has never before been considered. Work on other DPN-linked enzyme systems than ADH is in progress. It is interesting to recall that Hellerman, Lindsay and Bovarnich²³ found *d*-aminoacid oxidase to be inhibited by *p*-chloromercuribenzoic acid in competition with the prosthetic group, flavineadenine-dinucleotide. A

similar mechanism thus seems to operate in this flavine enzyme as in the ADH-DPN.

Evidently the linkage HS- to pyridine can stabilize the bond between DPNH and ADH, so that the dissociation constant of the DPNH · ADH becomes 200 times lower than for DPN · ADH at pH 7. It is further to be expected that this difference would be diminished at higher pH for the following reasons. As will be shown experimentally in part II, the dissociation constant, D_{red} , of the ADH · DPNH is 10^{-7} at pH 7 and 3×10^{-6} at pH 10. The D_{ox} moves the opposite way with pH, from 2×10^{-5} at pH 7 to a lower value near 3×10^{-6} at pH 10.

Meyerhof, Ohlmeyer and Möhle²⁴ found that DPN forms addition compounds with cyanide and bisulfite, presumably of the formulas



The reactions were reversible and, in the case of bisulfite, rapid. "Bisulfit reagiert so rasch dass in seiner Gegenwart die enzymatische Reoxydation von CzH₂ spektroskopisch nicht gemessen werden kann." The absorption band in the near ultraviolet had its maximum at 320 $m\mu$ instead of 340 $m\mu$. Our DPNH-ADH complex may thus be analogous with the bisulfite-DPN addition product.

It would be tempting to speculate upon the different possibilities implied in these observations. However, we still lack information on the amino acid composition and the nature of the sulfur compounds in ADH. Some preliminary experiments showed that simple sulfhydryl compounds like thioglycolic acid, cysteine, and glutathione do not give any spectroscopically observable addition compounds with DPN at pH 7–10. The nitroprussid test remained negative after the addition of an equivalent amount of DPNH to ADH at pH 9.5.

DPN and DPNH may further be assumed to be linked by way of their phosphoric acid components to some unknown group or groups in the ADH. This linkage, however, would probably not be influenced to any great extent by the oxidation state of the pyridine component.

Much more work has to be done in order to elucidate the chemical mechanism of the interaction between ADH, DPN and alcohol. Such work on the basis of the observations presented above is in progress.

THE PHYSIOLOGICAL OXIDATION OF ALCOHOL

Ethanol is combusted in the human body, mainly in the liver, at a rate of 7 g per hour, at 70 kg body weight. The rate is independent of the ethanol concentration down to the limit of the Widmark test, around 0.1 ‰ in the blood²⁶. In terms of micromolarity this figure corresponds to 2 000 μM , around three times the K_m at physiological pH. It is thus obvious that a rectilinear course of the physiological ethanol combustion is to be expected from the kinetic data down to values too low to be determined by the Widmark method, under the assumption that the k_3 of human DPNH-ADH is not largely different from horse DPNH-ADH. The content of ADH in the horse liver can now be calculated under the assumption that enough of free DPN is available to give maximal turnover of ethanol. If 1 molecule of ADH, $M = 73\ 000$, turns over 140 mols of ethanol per minute, and the observed rate is 7 g ethanol per hour, 1.32 g ADH would be required. This figure agrees very well with the value estimated from the preparative yields, about 1 g per kg liver, since a human liver weighs about 1.5 kg. It thus seems reasonable to assume that the ADH content will be about the same in horse and human liver. The turnover number is very probably higher at 37° than at 20°, so that less ADH would be required, but on the other hand it is not probable that the ADH works quite at maximal velocity, with excess of DPN, under physiological conditions. These effects would more or less compensate one another.

The results discussed above seem to give convincing evidence to prove that ADH is the enzyme essentially responsible for the physiological combustion of ethanol. The possibility of an oxidation of alcohol by catalase and hydrogen peroxide has been considered by Chance²⁷. In the case of ethanol, such a mechanism may not play any great role because the rectilinear course of ethanol oxidation is scarcely compatible with the catalase kinetics. On the other hand the physiological oxidation mechanism of methanol actually proposed by Chance²⁷, is rendered more probable by these data which show no methanol activity for liver ADH.

SUMMARY

1. The molecular weight of horse liver alcohol dehydrogenase (ADH) is = 73 000, that of yeast alcohol dehydrogenase is around twice as high.
2. When increasing quantities of ADH are added to DPNH, the absorption maximum shifts from 340 to 325 $m\mu$ and becomes somewhat lower. There is an isobestic point for free and ADH-bound DPNH at 328 $m\mu$. The millimolar extinction coefficients for both forms in the region 310 to 350 $m\mu$ are given.

3. By the aid of these extinction differences it was possible to establish that two molecules of DPNH are bound to one molecule of ADH from pH 7—9, at pH 10 around 1 DPNH/1 ADH.

4. The equilibrium constant

$$K = \frac{[\text{DPNH}] [\text{CH}_3\text{CHO}] [\text{H}^+]}{[\text{DPN}] [\text{C}_2\text{H}_5\text{OH}]}$$

was found to be raised on the addition of increasing amounts of ADH to new levels when ADH was in excess over DPNH. K became around 200 times higher at pH 6.4—7.8, around 20 times higher at pH 9, and only slightly higher at pH 10.

5. This increase in K is due to the DPNH being more firmly bound to ADH than DPN. The redox potentials of the coenzyme-enzyme complex were calculated from the equilibrium data and were found to approach the level of the potentials for the ethanol-acetaldehyde. The conclusion is drawn that the coupling of DPN and DPNH to ADH greatly facilitates the oxidation of ethanol to acetaldehyde.

6. Data for the Michaelis constants and the maximal reaction velocities, and their variations with pH are given.

7. The substrate specificity of the ADH was studied. It is especially interesting that the ADH-system does not react with methanol, but with many higher primary alcohols, including vitamin A.

8. *p*-Chloromercuribenzoic acid inhibits the enzyme activity (G. Wald and R. Hubbard). We found the same reagent to cause an immediate shift of the 325 $m\mu$ band of the DPNH-ADH complex to 340 $m\mu$. It is concluded that sulfhydryl groups of the ADH are linked to DPNH in the active complex, most probably to the pyridine ring, since the whole absorption band derives from this part of the molecule. This seems to be the first observation of pyridine nucleotides being activated by enzyme SH-groups.

9. The concentration of ADH in the liver, around 1 g per kg wet weight, fits very well to the known rate of the physiological ethanol oxidation. The Michaelis constant gives an explanation of its rectilinear course with time. It is concluded that the ADH-system, and not catalase + H_2O_2 , is the physiological catalyst for ethanol oxidation.

The authors' thanks are due to the Rockefeller Foundation, to Svenska Malt-drycksforskningsinstitutet and Stiftelsen Therese och Johan Anderssons Minne for grants.

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Received April 28, 1951.