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Studies on Liver Alcohol Dehydrogenase

II. The Kinetics of the Compound of Horse Liver Alcohol Dehydrogenase and Reduced Diphosphopyridine Nucleotide

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The elucidation of the mechanism of enzyme action by means of direct measurements of enzyme-substrate compounds has previously been limited to the peroxide compounds of the hemoproteins [1]. In catalases and peroxidases, the addition of substrate causes changes of absorption spectra that are readily measurable with rapid and sensitive spectrophotometric methods [2]. Theorell and Bonnichsen [3] have recently found a small shift in the spectrum of reduced diphosphopyridine nucleotide (DPNH) upon the addition of liver alcohol dehydrogenase (ADH) **. This paper describes kinetics and equilibrium studies of the enzyme-substrate compound, ADH-DPNH, in the reduction of aldehyde (Ald) to alcohol (Alc). The reactions of this enzyme-substrate complex are outlined by the following equations,

$$\text{ADH} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ADH-DPNH} \qquad (1)$$

$$\text{H}^+ + \text{ADH-DPNH} + \text{Ald} \underset{}{\overset{k_4}{\rightleftharpoons}} \text{ADH-DPN}^+ + \text{Alc} \qquad (2)$$

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+ \qquad (3)$$

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** The symbol ADH is here taken to represent the portion of the alcohol dehydrogenase molecule that binds one molecule of DPNH.

and the assumptions and simplifications represented by these equations are discussed below. The values of the equilibrium constant for Eqn. 1, and for the values of the reaction velocity constants, k_1 and k_4, are determined experimentally and are compared with values computed from studies of the overall activity of this enzyme.

PREPARATIONS

ADH and DPNH were prepared as described by Bonnichsen[4, 5] and had purities of 70—100 % and 69 % respectively. The actual concentration of ADH was computed according to part I.

The concentration of acetaldehyde was tested enzymatically with ADH and excess DPNH at pH 7.0. The concentration of formaldehyde was tested by the method of MacFadyen[6].

METHODS

The spectroscopic data of Theorell and Bonnichsen show that the largest changes of molecular extinction coefficient ($\Delta\varepsilon$) of the DPNH spectrum caused by the addition of ADH occur at 310 and 350 mμ. The actual values of $\Delta\varepsilon$ are small, 1.5 and 2.4 $cm^{-1} \times mM^{-1}$ at 310 and 350 mμ respectively. There is an isosbestic point between the DPNH and ADH-DPNH spectra (after subtraction of the ADH absorption) at 328 mμ. But there is no single wavelength where the formation of ADH-DPNH may be recorded without recording changes in [DPNH] at the same time.

In equilibrium studies of the ADH-DPNH complex (part I) it is possible to correct for the DPNH or ADH absorption, but it is neither practical nor accurate to do this in kinetic studies.

Thus the problem of measuring the reaction kinetics of the ADH-DPNH complex is a much more formidable one that that of measuring the hydrogen peroxide compounds of catalases and peroxidases. In the latter case the substrate has negligible absorption in the region of the enzyme and thus the measurements of the enzyme kinetics are not interfered with. In addition the molecular extinction coefficients of catalases and peroxidases change by about 50 $cm^{-1} \times mM^{-1}$ at 405 mμ on combination with peroxide. In the case of the ADH · DPNH complex, the change of extinction coefficient is much less than that of the hemoproteins, namely 2.4 $cm^{-1} \times mM^{-1}$ at 350 mμ and the DPNH absorption is relatively large at this wavelength ($\varepsilon_{350} = 5.7$ $cm^{-1} \times mM^{-1}$). It has been necessary to develop a spectrophotometric method that will respond only to the formation and disappearance of the ADH-DPNH complex and reject the changes of light absorption caused by DPNH alone.

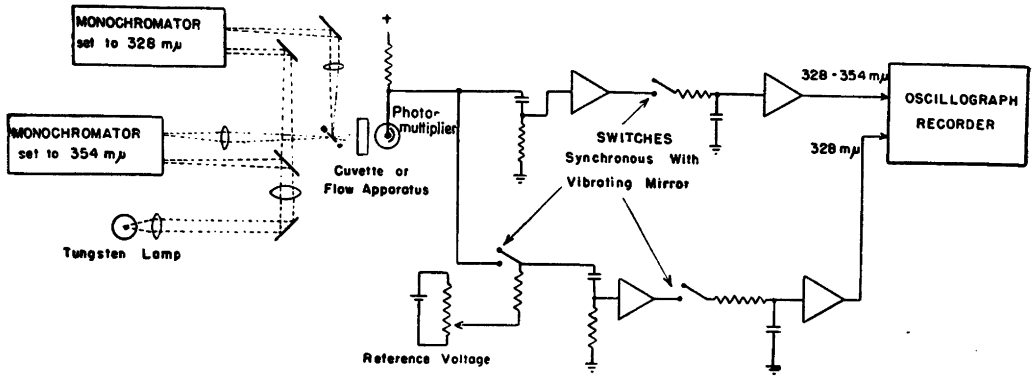

Fig. 1. A block diagram of the double beam system for measuring the difference of light absorption at 328 and 354 mμ as well as the light absorption at 328 mμ only. All parts of the optical system, except the lamp bulb, are quartz. The mirror and the switches vibrate at 60 cps. The symbol —▷— represents an amplifier. (MD-13.)

A consideration of the spectrum of DPNH shows that the extinction coefficients of DPNH are very nearly equal at the wavelengths 328 and 354 mμ. Thus a spectrophotometric method that measures only the difference of light absorption at these two wavelengths effectively rejects changes in concentration of DPNH. On the other hand the difference of extinction coefficients of the ADH-DPNH complex at these two wavelengths is measurable (about 1.4 $cm^{-1} \times mM^{-1}$ — see Table 1 of part I).

By alternately flickering light of a wavelength of 328 and 354 mμ through the ADH + DPNH solution and thence to a photocell, an alternating current wave is obtained whose amplitude represents only the difference of light absorption caused by the ADH-DPNH complex. And by more complex electronic circuits [2], it is possible to record simultaneously the change of light absorption at 328 mμ which is a measure of the [DPNH] since the ADH-bound and the free DPNH spectra have an isosbestic point at this wavelength. Fig. 2 illustrates the performance of this circuit. The lower trace indicates the deflection recorded by the circuit operating at 328 mμ when buffer alone and buffer plus 8.4 μM DPNH are alternately placed in the light path. The upper trace shows that the change of [DPNH] causes a change of only 1 % in the circuit measuring the difference of light absorption at 328 and 354 mμ. The wavelength readings for optimum rejection vary about ± 0.5 mμ. Since the wavelengths must be set accurately in order to achieve good rejection of DPNH, an experimental test similar to that of Fig. 2 is made before each experiment and the longer wavelength is adjusted to give optimum rejection (see Fig. 3). Thereby thermal drift of the monochromators is eliminated.

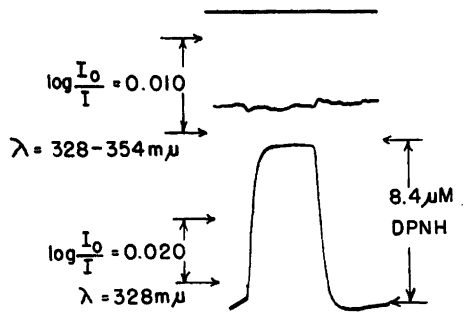

Fig. 2. The measurement of 8.4 μM DPNH by the optical density change at 328 mμ (lower trace) and the nearly complete rejection of this optical density change by a differential measurement between 328 and 354 mμ (upper trace) (Expt. 7b).

Under actual experimental conditions the ratio [DPNH] : [ADH] rarely exceeds 2 and the error in the measurement of [ADH · DPNH] caused by [DPNH] is about 2 %.

The sensitivities used in Fig. 2 are representative of those necessary for the measurement of the equilibrium and kinetics of the reactions of ADH and DPNH. The random fluctuations of the upper trace correspond to an optical density increment (log I_0/I) of 5×10^{-4}. In view of the small errors caused by random fluctuations and by the change of [DPNH], it is possible to use concentrations of ADH and DPNH as low as a few micromolar.

A typical record of the titration of dilute ADH with DPNH is given in Fig. 3. In this case an open 3 ml cuvette is used and 0.01 ml additions of 0.63 *mM* DPNH are made with a stirring rod. The first deflection of the traces only represents the stirring of the solution and it is seen that no net change of optical density results. Each of the successive deflections (except the last

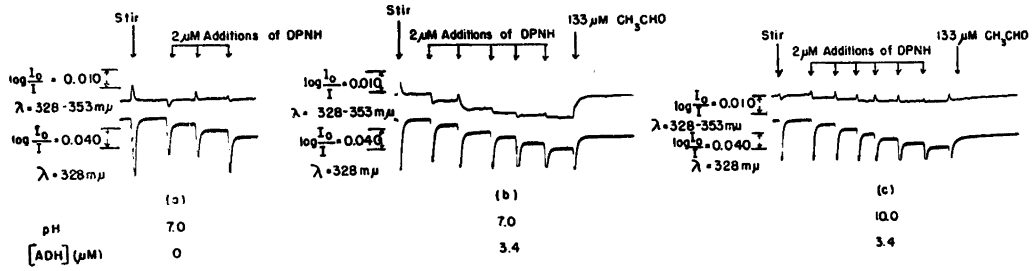

Fig. 3. Illustrating the titration of ADH with DPNH. The difference between the absorption at 328 and 354 mμ is recorded on the upper trace and the absorption at 328 mμ is recorded on the lower trace. Record a is a control experiment by which the rejection of DPNH by the circuit operating at 328—354 mμ is tested with [ADH] = 0. A downward deflection represents an increase in light absorption at 328 mμ and a decrease of absorption at 328—354 mμ. 3.4 μM ADH, pH = 7.0, 0.01 M PO_4''', pH = 10, 0.01 M glycine-NaOH buffers. (Expt. 5c, 33c—35.)

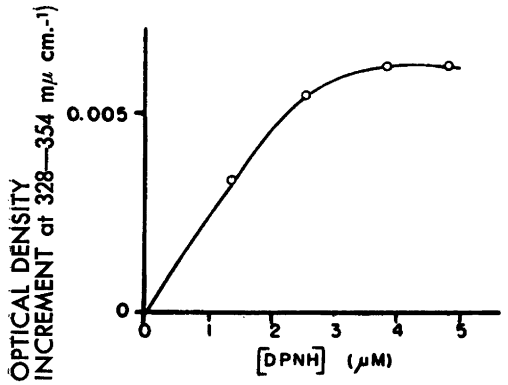

Fig. 4. The results of the titration of very dilute ADH with DPNH at pH = 7.0. The performance of the apparatus used in this experiment is given by Fig. 2. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4''' buffer. (Expt. 6f.)

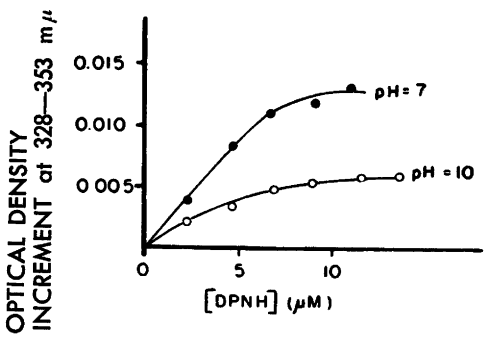

Fig. 5. The results of a titration of ADH with DPNH at pH = 7 and 10 plotted from the experimental data of Fig. 3. (Expt. 5c, 33c—35.)

one) correspond to the addition of 2 μM DPNH (0.01 ml of 0.6 mM) and it is seen that the deflections increase regularly at 328 mμ. In fact the exact amount of DPNH added is determined from this trace. In record (a) no ADH is present and the addition of DPNH causes no effect upon the circuit recording at 328—353 mμ. In record (b) 3.4 μM is present and differences between the absorption at 328 and 353 mμ are now recorded. On the first few additions of DPNH, a relatively large decrease of optical density is measured, but further additions cause relatively smaller increases. On the last addition, acetaldehyde (133 μM) is added which reacts with the ADH-DPNH complex and liberates ADH, causing the trace at 328—353 mμ to return to the original base-line. These records are discussed in detail below.

It was found that the combination of ADH with DPNH is too fast to be measured by ordinary mixing methods and a special flow apparatus having a rectangular observation tube with an optical path of 1 cm was used. Control experiments on the rate of formation of the peroxidase hydrogen peroxide complex show that the mixing is adequate for the measurement of reactions whose half-times are greater than 0.01 sec [2]. By using very dilute ADH and DPNH the half-times of the reactions actually measured were of the order of 0.1—0.05 sec for which this type of apparatus performs satisfactorily.

In summary, it may be said that the measurement of the kinetics and equilibrium of ADH and DPNH has been made possible by new spectrophotometric and rapid reaction techniques that have been used up to the limit of

their sensitivity and time resolution. The accuracy of the results is therefore not as great as might be desired but is surely adequate for a preliminary exploration of the mechanism of the reaction.

THE EQUILIBRIUM OF DPNH AND ADH

The titrations reported earlier in part I were necessarily carried out at such high concentrations of ADH and DPNH that no measurable dissociation of DPNH occurred. Those experiments were, however, excellently suited to a determination of the stoichiometry of the ADH-DPNH reaction. With the more sensitive techniques described above it is possible to reduce the [ADH] by a factor of 10 to 100 and Fig. 4 shows that a measurable dissociation constant can be obtained when 1.17 μM ADH (see footnote p. 1127) is titrated with DPNH. The average of the two values of dissociation constant computed from Fig. 4 is about 10^{-7} M (based on the fact that ADH binds 2 DPNH at pH = 7.0 (part I). The affinity of ADH for DPNH is indeed very high.

The effect of pH upon the optical density change caused by the formation of the ADH-DPNH complex is shown by the original data of Fig. 3 which are plotted in Fig. 5. Since somewhat larger [ADH] must be used to give adequte deflections at pH = 10, the titration at pH = 7.0 gives a scarcely measurable dissociation constant. But the value of the dissociation constant at pH = 10 can be calculated to be roughly 3×10^{-6} M (based on the fact that ADH binds 1 DPNH at pH = 10 (part I). Thus there is an increased dissociation of DPNH from ADH in alkaline solutions.

THE RELATIONSHIP BETWEEN THE ADH-DPNH COMPLEX AND THE REDUCTION OF ALDEHYDE

The experiment in Fig. 3 at pH = 7 shows that the ADH-DPNH complex rapidly disappears upon the addition of acetaldehyde. In fact, the reaction with acetaldehyde is so rapid that it has been difficult to increase the acetaldehyde concentration much above a stoichiometric equivalent of DPNH without causing the life-time of the ADH-DPNH complex to be too short for accurate measurement. Fortunately, it has beens found that formaldehyde reacts with the ADH-DPNH complex much more slowly than acetaldehyde and therefore the most satisfactory kinetic experiments have been carried out on the reduction of formaldehyde by the system ADH + DPNH.

A typical example of the formation and disappearance of the ADH-DPNH complex in the presence of excess formaldehyde is given in Fig. 6. As usual, the disappearance of DPNH is measured simultaneously at 328 mμ. These

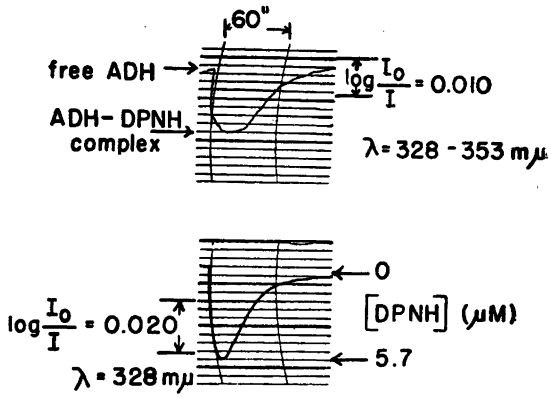

Fig. 6. The kinetics of formation and disappearance of the ADH · DPNH complex measured at 328—354 mμ (upper trace) and the simultaneous disappearance of DPNH measured at 328 mμ (lower trace). 1 cm optical path flow apparatus. 1.17 μM ADH, 8.8 μM DPNH (initial), 66 μM HCHO pH = 7.0, 0.01 M PO_4'''. (Expt. 7c—5.)

experiments were carried out in the flow apparatus by mixing ADH with DPNH plus HCHO. Since the experiment shown in Fig. 6 is the second of a duplicate set, the record begins with "free ADH" (+DPN) remaining from the previous test. On initiating the flow of reactants, the fairly rapid formation of the ADH-DPNH complex starts immediately as is indicated by the fall of the 328—354 mμ trace. The complex exists in a steady state for some seconds, and then decomposes into "free ADH". At 328 mμ, the initiation of the flow replaces the spent DPNH solution and causes the abrupt downward deflection (increase in optical density). The chemical reaction causing the disappearance of DPNH then proceeds and the linear upward sweep of the trace is sustained until the [DPNH] falls to a very low value.

One of the obvious criteria of an enzyme-substrate complex that follows the theory of Michaelis and Menten is that the half-time for the cycle of the enzyme-substrate complex should be twice the half-time for the overall reaction[7]. The values of the half times are 51 and 25 sec. for the cycle and the overall reaction respectively and, according to this criterion, verify the role of the ADH-DPNH complex to be that indicated by Eqns. 1 and 2.

The velocity constant for the reaction of ADH-DPNH with formaldehyde (k_4) is computed directly from experiments similar to those of Fig. 6 by measurement of the kinetics of the ADH-DPNH complex. The value of k_4 is computed according to the following equation (4)

$$k_4 = \frac{[\text{DPNH}]_0}{[\text{ADH} \cdot \text{DPNH}]_m \; t_{\frac{1}{2}\ \text{off}} \; [\text{HCHO}]_0} \tag{4}$$

$[\text{DPNH}]_0$ is the initial [DPNH] (M)
$[\text{HCHO}]_0$ is the initial [HCHO] (M)

Table 1. A summary of values for the velocity of the reaction of formaldehyde and acetaldehyde with the ADH-DPNH complex. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4'' 27° C, (Expt. 7c)
(note: 1.17 μM ADH gives 2.34 μM complex.)

$[DPNH]_0$ μM	8.8	3.7	7.4	7.4	$[DPNH]_0$ μM	7.5	15
$[HCHO]_0$ μM	66	66	132	330	$[CH_3CHO]_0$ μM	33	66
$[ADH\text{-}DPNH]_m$ μM	2.3	2.0	2.3	2.0	$[ADH\text{-}DPNH]_m$ μM	0.56	0.89
$t\frac{1}{2}$ off (sec.)	52	22	19	8.5	$t\frac{1}{2}$ off (sec.)	2	3
$k_4 \times 10^{-3}$ ($M^{-1} \times sec.^{-1}$)	1.1	1.3	1.3	1.3	$k_4 \times 10^{-5}$ ($M^{-1} \times sec.^{-1}$)	2	0.9

$[ADH\text{-}DPNH]_m$ is the maximum concentration of the complex formed in the particular experiment (M).* $t\frac{1}{2}$ off is the time interval between formation and half-disappearance of the ADH-DPNH complex (sec).

The value of k_4 for several experimental conditions is given in Table 1. The average value for formaldehyde is 1.3×10^3 $M^{-1} \times sec^{-1}$ at pH = 7.0.

Considerable difficulty was experienced in obtaining satisfactory "cycles" of the ADH-DPNH complex in the presence of a reasonable excess of acetaldehyde over DPNH for under those conditions the complex concentration ($[ADH\text{-}DPNH]_m$) is too small for accurate measurement. The preliminary value of k_4 for acetaldehyde based on the data of Table 1 is 10^5 $M^{-1} \times sec^{-1}$.

THE VELOCITY CONSTANT FOR THE FORMATION OF THE ADH-DPNH COMPLEX

The rapid formation of the ADH-DPNH complex is verified by records such as Fig. 6 which show that ADH and DPNH even in extremely dilute solution combine in much less than 1 sec. The flow method has therefore been used and a typical record is shown in Fig. 7. Formaldehyde is present so that the baseline before starting the flow corresponds to the optical density of free ADH. The flow is then started momentarily and the formation of the complex is observed. The flow is then restarted and is held near its maximum velocity for over one second in order to allow the photoelectric circuits to respond

* The concentration of ADH used in this computation is twice the actual molarity of ADH because two molecules of DPNH are bound to one molecule of ADH at pH = 7.0[3].

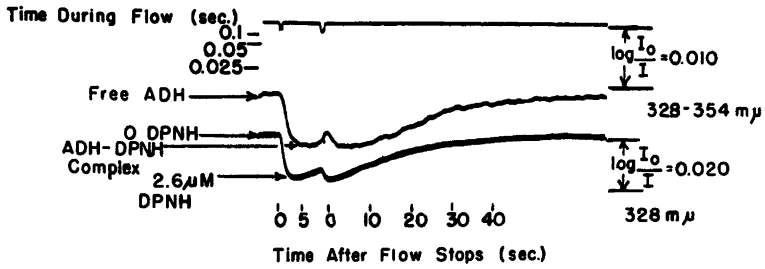

Fig. 7. A measurement of the speed of combination of ADH and DPNH. 1 cm path flow apparatus. 1.17 μM ADH, 3.7 μM DPNH, 66 μM HCHO, pH = 7.0, 0.01 M PO_4'''. (Expt. 7c—10.)

fully to the change of optical density. The trace clearly indicates that the maximum amount of complex is not reached during the flow. As soon as the flow stops, the maximum amount of complex is formed.

The trace at 328 mμ serves as a good control, in this case the second initiation of the flow should only restore the [DPNH] to its full value — and so it does.

The velocity constant for the formation of the ADH-DPNH complex (k_1) may be computed according to the second order equation on the assumption that the two DPNH molecules are bound independently and that the effective molarity of ADH is twice its actual concentration

$$k_1 = \frac{2.3}{t([\text{DPNH}]_0 - [\text{ADH}]_0)} \log \frac{[\text{ADH}]_0([\text{DPNH}]_0 - [\text{ADH-DPNH}]_t)}{[\text{DPNH}]_0([\text{ADH}]_0 - [\text{ADH-DPNH}]_t)} \quad (5)$$

The subscript 0 denotes initial concentrations and t denotes the concentration at time t.

A summary of the values of k_1 obtained with several values of $[\text{DPNH}]_0$ is given in Table 2. The average of all values is $k_1 = 4 \times 10^6$ M^{-1}, $\times$ sec^{-1}, although the values obtained at higher [DPNH] are considered less accurate.

DISCUSSION

These experiments show that the combination of DPNH with liver alcohol dehydrogenase is a rapid reaction and that the DPNH is tightly bound to the protein. An estimate of the value of the velocity constant for the dissociation of DPNH molecules from the protein is given by the product of the dissociation constant and the velocity constant for the combination of DPNH and ADH

Table 2. A summary of data on the velocity constant for the combination of ADH and DPNH. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4'', 27° C, (Expt. 7c) (note: 1.17 μM ADH gives 2.34 μM complex).

$[DPNH]_0$ (μM)	4.3	3.7	7.4	7.4
$[ADH\text{-}DPNH]_m$ (μM)	1.6	1.9	1.7	2.0
t (sec.)	0.067	0.12	0.12	0.095
$k_1 \times 10^{-6}$ ($M^{-1} \times sec.^{-1}$)	5.2	5.4	1.6	3.2

($4 \times 10^6 \times 10^{-7} = 0.4$ sec.$^{-1}$). Thus the half-time for the dissociation reaction is about 1.7 sec. $(\frac{0.693}{0.4})$.

The complex of ADH and DPNH also reacts fairly rapidly with aldehydes, the velocity constants are 1.3×10^3 and 10^5 $M^{-1} \times sec^{-1}$ for formaldehyde and acetaldehyde respectively.

The results of these kinetic tests may be summarized according to the following equations for the case of acetaldehyde:

$$\text{ADH} + \text{DPNH} \underset{k_2 = 0.4 \text{ sec}^{-1}}{\overset{k_1 = 4 \times 10^6 M^{-1} \times sec^{-1}}{\rightleftharpoons}} \text{ADH-DPNH} \qquad (6)$$

$$\text{ADH} \cdot \text{DPNH} + \text{Ald} + H^+ \overset{k_4 \approx 10^5 \ M^{-1} \times sec^{-1}}{\rightleftharpoons} \text{ADH-DPN}^+ + \text{Alc} \qquad (7)$$

The velocity constant k_4 represents the value for the reaction of ADH-DPNH and aldehyde at pH = 7.0.

At pH = 7, the reaction of alcohol and ADH-DPN is very slow and is neglected.

In the presence of various small formaldehyde concentrations, the values of k_4 are reasonably constant and it is probable that the dissociation of DPN from the complex is not a rate limiting step with dilute formaldehyde. In this case the reaction mechanism is identical to that previously studied for catalases and peroxidases:

$$E + S \rightleftharpoons ES \qquad (8)$$

$$ES + A \rightleftharpoons E + P \qquad (9)$$

and the methods of computing k_4 derived previously are valid [8].

When more concentrated aldehyde solutions are used an accumulation of the ADH-DPN complex is to be expected. Studies of the overall reaction are in accord with this explanation because a definite maximum in the aldehyde-activity relationship is obtained [3]. Although such a maximum might be attributed to an ADH-aldehyde complex, the accumulation of the ADH-DPN complex would provide an equally satisfactory explanation. The next step in the reaction is then assumed to be

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+ \tag{10}$$

In order to determine whether the mechanism outlined by the Eqns. 6, 7 and 10 is compatible with the observed data on the overall activity of ADH, it is necessary to determine the relationships between the reaction velocity constants as defined by these equations and the values of activity and the "Michaelis constants" for DPNH and acetaldehyde. Eqns. 6, 7, and 10 are comverted into the usual form for computations of enzyme-substrate kinetics:

$$\begin{array}{ccccc} \text{E} & + \text{S}_\text{I} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \text{ES}_\text{I} & \\ e - p_1 - p_2 & x_1 & & p_1 & \end{array} \tag{11}$$

$$\begin{array}{ccccccc} \text{ES}_\text{I} & + & \text{A} & \overset{k_4}{\rightleftharpoons} & \text{ES}_\text{II} & + & \text{AH} \\ p_1 & & a_1 & & p_2 & & a_2 \end{array} \tag{12}$$

$$\begin{array}{ccccc} \text{ES}_\text{II} & \overset{k_3}{\rightleftharpoons} & \text{E} & + & \text{S}_\text{II} \\ p_2 & k_5 & e - p_1 - p_2 & & x_2 \end{array} \tag{13}$$

In this form the first two steps of the reaction mechanism are seen to be a form of the simple mechanism shown to apply, for example, to catalase, alkyl-hydrogen peroxides, and alcohol [9]. But in this case the enzyme is not released until ES_II is dissociated. As usual, the concentrations of the various reactants at any time are written underneath the appropriate symbols. Since only a steady state analysis is required, only the differential equations for p_1, p_2, and a are required.

$$\frac{dp_1}{dt} = k_1\ x_1\ (e - p_1 - p_2) - (k_2 + k_4a_1)\ p_1 \tag{14}$$

$$\frac{dp_2}{dt} = k_4a_1p_1 - k_3p_2 + k_5x_2\ (e - p_1 - p_2) \tag{15}$$

$$\frac{da_1}{dt} = k_4a_1p_1 \tag{16}$$

In the steady-state both $\frac{dp_1}{dt}$ and $\frac{dp_2}{dt}$ are negligible and p_1 and p_2 can be computed.

$$p_1 = \frac{k_1\ x_1\ (e - p_2)}{k_1x_1 + k_2 + k_4a_1} = \frac{e - p_2}{1 + \frac{K_{m_1}}{x_1}} \tag{17}$$

$$K_{m1} = \frac{k_2 + k_4a_1}{k_1} \tag{18}*$$

$$p_2 = \frac{k_4\ a_1p_1 + k_5\ x_2\ (e - p_1)}{k_3 + k_5x_2} = \frac{e - p_1\,(1 - \frac{k_4a_1}{k_5x_2})}{1 + \frac{K_{m_2}}{x_2}} \tag{19}$$

$$K_{m_2} = \frac{k_3}{k_5} \tag{20}$$

K_{m_2} is actually the dissociation constant of the ADH-DPN complex and is about 2×10^{-5} M. See below p. 1141.

$$\frac{p_1}{e} = \frac{1}{(1 + \frac{x_2}{K_{m_2}})\ (1 + \frac{K_{m_1}}{x_1}) - \frac{x_2}{K_{m_2}} + \frac{k_4a_1}{k_3}} \tag{21}$$

The overall activity is usually measured by the rate of disappearance of DPNH, $-\frac{da_1}{dt}$, and the turnover number is $\frac{1}{e} \times \frac{da_1}{dt}$
From Eqn. 16,

* K_{m_1} is usually regarded as a Michaelis constant.

$$\frac{1}{e} \times \frac{da_1}{dt} = k_4 a_1 \times \frac{p_1}{e} \tag{22}$$

and on substituting for $\frac{p_1}{e}$ its value given in Eqn. 21,

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{k_4 a_1 [(1 + \frac{x_2}{K_{m_2}})\ (1 + \frac{K_{m_1}}{x_1})] - \frac{x_2}{K_{m_2}} + \frac{1}{k_3}} \tag{23}$$

Under the conditions of these experiments a very small [DPN] is formed since the initial [DPNH] is very low. And in studies of the overall activity, the initial rate is measured (part I) and again very little [DPN] forms. Thus the amount of DPN under both these conditions is probably negligible compared to K_{m_2} ($\sim 1 \times 10^{-5}$ M). Eqn. 23 is therefore simplified as follows

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_4 a_1}(1 + \frac{k_2}{k_1 x_1}) + \frac{1}{k_3}} \tag{24}$$

The values of [DPNH] giving maximal turnover number then depends upon the [aldehyde]. For large [aldehyde],

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_3}} \tag{25}$$

and the maximum turnover number for large [DPNH] is

$$\frac{1}{e}\frac{da_1}{dt} = k_3 \tag{26}$$

The [DPNH] giving half maximal activity in the presence of excess aldehyde is

$$(x_1)_{\frac{1}{2}} = \frac{k_3}{k_1} \tag{27}$$

Thus at *high* [aldehyde], the "Michaelis constant"for DPNH depends upon the rate of dissociation of DPN from ADH, and not at all upon the rate of dissociation of DPNH from ADH.

For *low* [aldehyde] ($k_3 \gg k_4 a$)

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_4 a_1}\left(1 + \frac{k_2}{k_1 x_1}\right)} \tag{28}$$

and the maximum turnover number is

$$\frac{1}{e}\frac{da_1}{dt} = k_4 a_1 \tag{29}$$

The [DPNH] giving half maximal activity in the presence of low [aldehyde] is

$$(x_1)_{\frac{1}{2}} = \frac{k_2 + k_4 a_1}{k_1} = K_{m_1} \tag{30}$$

Thus only at very low [aldehyde] will the true dissociation constant for the ADH-DPNH complex be measured (when $k_2 \gg k_4 a_1$).

At large [DPNH], the effect of aldehyde upon the activity will be as follows:

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_4 a_1} + \frac{1}{k_3}} \tag{31}$$

For large [aldehyde] and [DPNH], the maximum turnover number is k_3 as already given by Eqn. 26. The [aldehyde] giving half maximal activity is

$$(a_1)_{\frac{1}{2}} = \frac{k_3}{k_4} \tag{32}$$

These formulas and the relevant assumptions are summarized in Table 3.

The values $(a_1)_{\frac{1}{2}}$ and $(x_1)_{\frac{1}{2}}$ corresponding to the conditions of Eqns. 27 and 32 as well as the value of k_3, have already been published by Theorell and Bonnichsen, see Table 7 part I.

Since no overall data are available on the reactions of ADH, DPNH, and formaldehyde, some new data are plotted in Fig. 8 and are summarized

Table 3. Summary of equations for calculation of reaction velocity constants (x_2, $a_2 \approx 0$).

Equation number	26	27	29	30	31	32
x_1	∞	$\frac{k_3}{k_1}$	∞	$\frac{k_2 + k_4 a_1}{k_1}$	∞	∞
a_1	∞	∞	small	small	variable	$\frac{k_3}{k_4}$
$\frac{1}{e}\frac{da_1}{dt}$	k_3	$\frac{k_3}{2}$	$k_4 a_1$	$\frac{k_4 a_1}{2}$	$\frac{1}{\frac{1}{k_4 a_1} + \frac{1}{k_3}}$	$\frac{k_3}{2}$

in Table 4. The values of the reaction velocity constants k_1 and k_4 are computed from the overall data on acetaldehyde and formaldehyde and are compared with the values obtained by direct measurements of the reaction kinetics of ADH-DPNH in Table 5.

The agreement of the overall and direct data is especially close in the tests in which dilute formaldehyde was used. And this corresponds most closely to the conditions used in the direct studies of the ADH-DPNH complex. In view of the fact that there is over 100 fold difference in the [ADH] for the two studies, the agreement is considered to be satisfactory.

If the mechanism described here applies to the oxidation of alcohol by ADH and DPN, the formulae derived for the computation of the reaction velocity constants can be used. Although no direct kinetic measurements have been made on the ADH-DPN complex, the value of k'_3 for the alcohol-DPN system (the velocity constants for this system are designated by primes) should be the same as the value of k_2 for the ADH-DPNH. The value of k'_3 computed from the data of Theorell and Bonnichsen at pH = 6.8 is 1.1 sec^{-1} and is in fair agreement with our calculated value of k_2 (0.4 sec^{-1}).

The value of K_m of Eqn. 20 ($K_m = \frac{k'_3}{k'_5}$) has already been determined by Theorell and Bonnichsen (part I) to be about 200 times greater than the dissociation constant of the ADH-DPNH complex at pH = 7.0, thus $\sim 2 \times 10^{-5}$. Since k_3 is already known (see Table 7, part I, $k'_3 = 39$—45 sec^{-1} at pH = 7.0), k'_5, the velocity constant for the combination of ADH and DPN, is computed to be 2×10^6 $M^{-1} \times sec^{-1}$. This value is in remarkably good agreement with the value of k_1, the velocity constant for the combination of ADH and

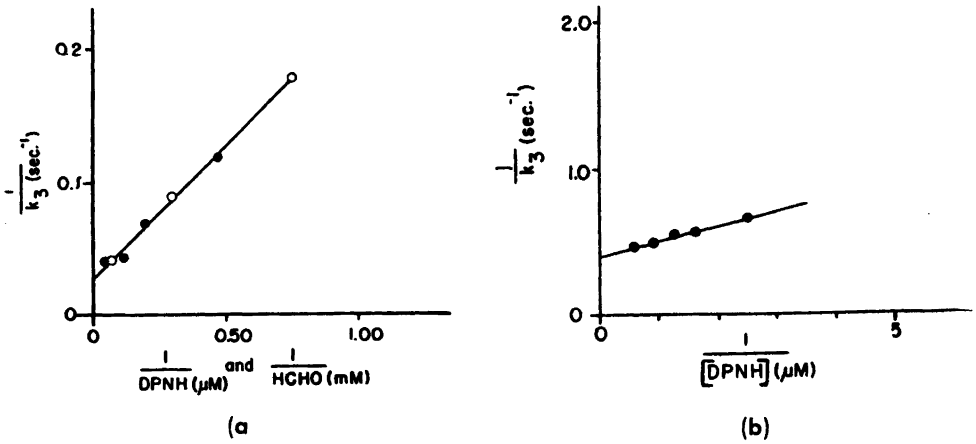

Fig. 8. The effect of [DPNH] and [HCHO] upon the activity of ADH. In Fig. 8 a the solid circles represent the values obtained with varying [DPNH] for [HCHO] = 10 mM. The open circles represent the values obtained with varying [HCHO] and [DPNH] = 8.6 μM. [ADH] = 3 × 10⁻⁹ M. In Fig. 8b, the effect of varying [DPNH] for 0.67 mM HCHO is shown. [ADH] = 15 × 10⁻⁹ M. pH = 7.22, 0.01 M PO_4''' for all experiments. (Expts. 9e, 9g.)

Table 4. Summary of overall data on the reaction of ADH, DPNH, and HCHO (pH = 7.22, 26°).

Substance	K_m (M)	k_3 (sec.$^{-1}$)	k_4a_1 (sec.$^{-1}$)	[HCHO] (mM)	[DPNH] (μM)
Formaldehyde	8×10^{-3}	19	—	—	8.6
DPNH	8×10^{-6}	19	—	10	—
DPNH	2.6×10^{-7}	—	1.25	0.67	—

DPNH, in view of the uncertainties of the various quantitites. The combination of ADH with DPN or DPNH apparently occurs at about the same speed, but their dissociation velocities differ considerably, DPNH being bound much more tightly. And on this basis the different activities of ADH towards alcohol and aldehyde are readily explained.

The close agreement of the reaction velocity constants for the combination of ADH with DPN and DPNH is reasonable in view of the similarity of the latter two molecules and in view of the probability that they combine at the

Table 5. Velocity constants for the reactions of ADH computed on the basis of the mechanism of equations 6, 7, and 10.

	Substrate used	k_1 ($M^{-1} \times sec.^{-1}$)	k_4 ($M^{-1} \times sec.^{-1}$)	k_2 ($sec.^{-1}$)
Computed from overall data	Acetaldehyde	1.6×10^6	2.2×10^5	—
	Formaldehyde (10 μM)	2.4×10^6	2.4×10^3	—
	Formaldehyde (0.67 μM)	4.8×10^6	1.9×10^3	—
	Alcohol	—	—	1.1
Measured directly from the kinetics of ADH-DPNH	Formaldehyde	4×10^6	—	0.4
	Formaldehyde	—	1.3×10^3	—
	Acetaldehyde	—	10^5	—

same position on the protein. The latter supposition is supported by preliminary experiments on the competition between DPN and DPNH for ADH.

There are many aspects of these reactions that require further study but these preliminary results encourage us to believe that the detailed analysis of the mechanism of action of catalases and peroxidase based on direct studies of enzyme-substrate compounds may be applied to the reactions of many enzyme systems.

SUMMARY *

1. A rapid spectrophotometric method for measuring the formation and disappearance of the compound of ADH and DPNH without appreciable interference from the absorption of DPNH has been developed.

2. A titration of very dilute ADH (1.17 μM) with DPNH gives a dissociation constant for the ADH-DPNH complex of 10^{-7} M at pH = 7.0.

3. The velocity constant for the formation of the ADH-DPNH complex is 4×10^6 $M^{-1} \times sec^{-1}$ at pH = 7.0.

4. The velocity constant for the dissociation of DPNH from ADH is computed to be 0.4 sec^{-1}.

5. The velocity constant for the reaction of the ADH-DPNH complex with formaldehyde is 1.3×10^3 $M^{-1} \times sec^{-1}$ at pH = 7.0. The values of k_4

* The experiments were carried out at 26° C.

are constant over a reasonable range of experimental conditions. A preliminary value for the velocity constant for acetaldehyde is 10^5 $M^{-1} \times sec^{-1}$ at pH = 7.0.

6. The ADH-DPNH complex in the presence of dilute formaldehyde fulfils the requirements for a Michaelis intermediate.

7. On the basis of the following mechanism for the action of ADH,

$$\text{ADH} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ADH} \cdot \text{DPNH}$$

$$\text{H}^+ + \text{ADH} \cdot \text{DPNH} + \text{Aldehyde} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \text{ADH-DPN}^+ + \text{alcohol}$$

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+,$$

the velocity constants k_1, k_4, and k_2 have been computed from data on the overall activity of the enzyme in very dilute solutions and values of 3×10^6 $M^{-1} \times sec^{-1}$, 2.2×10^5 $M^{-1} \times sec^{-1}$ (for acetaldehyde), 1.3×10^3 $M^{-1} \times sec^{-1}$ (for formaldehyde) and 1.1 sec^{-1} respectively are obtained and agree reasonably well with the values obtained by direct measurements of the kinetics of the ADH · DPNH complex. This agreement has been obtained without assuming the formation of compounds of ADH with aldehyde or alcohol.

8. The velocity constant for the combination of ADH and DPN is calculated to be about 2×10^{-6} M^{-1} sec^{-1} at pH = 7.0 and it is concluded that DPN and DPNH are bound by ADH at about the same speed and on the same place.

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