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Dissociation Constants of Ternary Complexes of Fatty Acids and Fatty Acid Amides with Horse Liver Alcohol Dehydrogenase-Coenzyme Complexes

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1. Fatty acids form dissociable binary compounds with liver alcohol dehydrogenase (ADH), and ternary compounds with DPN—ADH, but not with DPNH—ADH. The fatty acids compete with alcohols for the same binding site.

2. The amides of fatty acids form binary compounds with ADH, and ternary complexes with DPN—HADH, but not with DPN · ADH. They compete with aldehyde for the same binding site.

3. Spectrophotofluorometric methods were elaborated for determining all the dissociation constants involved: $K_{E,I}$; $K_{E.O}$; $K_{EO,I}$; and $K_{EI,O}$ in the case of fatty acids, $K_{E,I}$; $K_{E,R}$; $K_{ER,I}$; and $K_{EI,R}$ in the case of amides.

4. With increasing C-chain length the complex forming power increases for both acids and amides, in some of the combinations many thousand-fold. For the fatty acids this increase continues up to C_{15}. From C_{16} and upwards the complexing ability practically disappears.

5. In these ternary complexes coenzyme and substrate analog mutually stabilize one another's binding to the enzyme.

6. The high affinity of the long-chain fatty acids and amides for ADH—DPN or ADH—DPNH is parallelled by the high reaction velocities observed with higher alcohols and aldehydes in comparison with ethanol.

7. The significance of these results is discussed.

In 1955 Theorell *et al.*[1] presented data on the effects of anions on the liver-ADH ** system as determined from reaction velocity measurements. Strong effects with anions such as chloride, bromide, nitrate and sulfate were reported as had been previously observed from work on the old yellow enzyme [2]. Surprisingly, however, it was found that formate and acetate, which were inactive

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** Abbreviations, see p. 1730.

in the O.Y.E. system, had strong effects on the liver-ADH system. Formate was found to effect only two of the six velocity constants, *i. e.* DPN · ADH $\xrightarrow{k_2'}$ DPN + ADH and DPN · ADH + alcohol $\xrightarrow{k'}$ DPNH · ADH + + aldehyde + H^+. On this basis it was suggested that formate reacts with the DPN · ADH complex, but not with the reduced complex, DPNH·ADH. Since formate inhibited competitively with alcohol it was presumed that formate enters into the binding site for ethanol in DPN.ADH. The effect of acetate appeared to be weaker than for formate.

More recent work by Theorell and Winer [3] using the fluorometric technique to measure dissociation constants showed that while formate has a small apparent effect on $K_{E,R}$ it exerts a very strong apparent effect on $K_{E,O}$. This again suggested that the effect of formate depends on the formation of a ternary ADH · DPN · formate complex. Since the fluorometric method offers a rapid procedure for the determination of dissociation constants, it was decided to investigate the effects of other saturated fatty acids on the ADH-coenzyme system. The effects of fatty acid amides were also investigated in line with recent substrate specificity studies on liver ADH by one of us (A.D.W.) [4] which indicated that long chain aldehydes were, in some cases, better substrates than acetaldehyde.

It was found that all the fatty acids studied (C_1—C_{20}) form ternary complexes with ADH · DPN and compete with the ethanol binding site while all of the fatty acid amides studied (C_1—C_6) form ternary complexes with ADH · DPNH and compete with the aldehyde binding site. In this way fatty acids and amides act as inhibitors. Remarkably strong effects were found with increasing chain length for both acids and amides. From a suitable choice of experimental conditions it is possible to calculate the dissociation constants describing the effect of the inhibitor on ADH ($K_{E,I}$), on the ADH · coenzyme complex ($K_{ER,I}$ and $K_{EO,I}$) and of the coenzyme on the ADH · inhibitor complex ($K_{EI,O}$ and $K_{EI,R}$). It is suggested that the ADH · DPNH · amide and

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** ADH—DPN and ADH—DPNH, 1/2 molecule of horse liver alcohol dehydrogenase (E) and oxidized (O) or reduced (R) diphosphopyridine nucleotide, respectively.

E_t = Total conc. of liver alcohol dehydrogenase (ADH) (μN)
E = Conc. of free » » » » »
R_t = Total conc. of reduced diphosphopyridinenucleotide (DPNH)
R = Conc. of free » » (»)
O_t = Total conc. of oxidized » (DPN)
O = Conc. of free » » (»)
I_t = Total conc. of fatty acid or amide
I = Conc. of free » » » »

$$K_{E,R} = \frac{E \times R}{ER}$$

$K_{E,R,app}$ = apparent dissociation constant of ADH · DPNH in presence of I

$$K_{E,O} = \frac{E \times O}{EO}; \qquad K_{EI,R} = \frac{EI \times R}{ERI}$$

$$K_{E,I} = \frac{E \times I}{EI}; \qquad K_{EO,I} = \frac{EO \times I}{EOI}$$

$$K_{ER,I} = \frac{ER \times I}{ERI}; \qquad K_{EI,O} = \frac{EI \times O}{EOI}$$

ADH · DPN · fatty acid complexes, just like the fluorescent lactic dehydrogenase · DPNH · anion complexes recently described by Winer and Schwert [5], are similar in structure to activated complexes formed between ADH, DPNH, aldehyde and ADH, DPN, alcohol.

EXPERIMENTAL

Fluorometric measurements of emission spectra, the calculation of the dissociation constants $K_{E,R}$ and $K_{E,O}$, the buffer salts used and the purity of DPN and DPNH (Sigma) have been previously described [3]. Fatty acids, C_1-C_9, were reagent grade chemicals further purified by distillation under suitable pressures; $C_{10}-C_{20}$ acids were gifts of Professor E. Stenhagen, Uppsala, and had the following melting points in °C: C_{10} 31.2—32.0; C_{11} 28.0—28.5; C_{12} 43.7—43.9; C_{13} 41.5—41.7; C_{14} 54.5—54.9; C_{15} 59.5—59.8; C_{16} 62.5——62.9; C_{17} 61.3—61.4; C_{18} 69.6—69.8; C_{19} 68.4—68.5; C_{20} 75.2—75.3. These acids were solubilized after lyophilization of the potassium salts. The amides were products of the Eastman Kodak Company and recrystallized twice from water prior to use. Heart muscle lactic dehydrogenase and beef liver glutamic dehydrogenase were commercial preparations from C. F. Boehringer and Son, Mannheim.

Enzyme preparation. Crystalline ADH as prepared by the method of Bonnichsen and Brink [6] and treated with DPN to remove traces of endogenous ethanol as previously described [3] was used for all experiments. Liver ADH prepared in this manner is surprisingly stable for long periods of time (2—3 weeks at 2°). The concentration of the enzyme * in the fatty acid experiments was determined by calculations from $C\left(\frac{a''}{b''}-\frac{a'}{b'}\right)Z^2-(a''-a')\,Z+(b''+b')=0$ ** using $Q_{E,R}$*** = 13.5 as determined previously. Calculated values for enzyme concentration were 10—20 % lower than those determined from activity measurements at pH 10 using the assay method of Dalziel [7].

In the amide experiments the concentration of ADH was determined by titration with DPNH in the presence of excess *iso*butyramide. This method of determining enzyme concentration has been found very much superior to activity measurements because of the high Q and the low dissociation constant $K_{EI,R}$ (= 0.02 μM).

The enzyme concentration as determined by the amide procedure was also 10—20 % lower than that determined by activity measurements. The Q for different amides was determined by two methods:

1. Addition of a small amount of DPNH to an excess of ADH and amide (»direct» method).

2. Titration of ~1 μN ADH with 6—10 steps of ~0.5 μM DPNH, and in experiments where appreciable dissociation occurred (for instance with acetamide) calculation of Z ($= Q-1$) from [3]

$$C\left(\frac{a''}{b''}-\frac{a'}{b'}\right)Z^2-(a''-a')\,Z+(b''+b')=0$$

and extrapolation to $I=\infty$ by the aid of eqn. (14) ("calculated" method).

METHODS OF CALCULATION OF DISSOCIATION CONSTANTS

1. I = Fatty Acids

The experimental results were found to agree with the assumption that fatty acids form binary complexes EI with ADH, but not ternary ERI complexes. Thus

* By ADH concentrations the number of binding sites is meant (2 sites/mole from pH 6—9).

** See the previous publication [3] for derivation of this equation.

*** Q is the deflection ratio DPNH · ADH/DPNH as measured with our apparatus at 410 mμ when activated at 325—330 mμ.

$$K_{E,R} = \frac{(E_t - ER - EI)(R_t - ER)}{ER} \quad (1)$$

From the experiments in presence of I we obtain:

$$K_{E,R\ app} = \frac{(E_t - ER)(R_t - ER)}{ER} \quad (2)$$

Only EI is unknown. Therefore

$$EI = (1 - K_{E,R} / K_{E,R\ app})\ (E_t - ER), \text{ and} \quad (3)$$

$$K_{E,I} = \frac{(E_t - ER - EI)(I_t - EI)}{EI} \quad (4)$$

When mixtures of ADH, I and DPN were titrated with DPNH, the experimental results were found to agree with the assumption that besides EI and ER also EO and EOI were formed.

$$K_{E,R} = \frac{(E_t - ER - EO - EI - EOI)(R_t - ER)}{ER} \quad (5)$$

$$K_{E,O} = \frac{(E_t - ER - EO - EI - EOI)(O_t - EO - EOI)}{EO} \quad (6)$$

$$K_{E,I} = \frac{(E_t - ER - EO - EI - EOI)(I_t - EI - EOI)}{EI} \quad (7)$$

ER is determined experimentally, EO, EI and EOI are unknown.

$K_{E,R}$ and $K_{E,O}$ are determined separately in experiments without I [3], $K_{E,I}$ from eqns 3 and 4.

Since O in these experiments was always much larger than EO and EOI, from eqns 5 and 6:

$$EO = \frac{K_{E,R} \times ER \times O}{K_{E,O}\ (R - ER)} \quad (8)$$

From eqns 6 and 7 when $I \gg EI + EOI$, $O \gg EO + EOI$:

$$EI = \frac{EO \times K_{E,O} \times I}{O \times K_{E,I}} \quad (9)$$

$$EOI = E_t - ER - EO - EI - \frac{ER \times K_{E,R}}{R_t - ER} \text{ (where the last term} = E) \quad (10)$$

$$K_{EO,I} = \frac{EO(I_t - EI - EOI)}{EOI} \quad (11)$$

$$K_{EI,O} = \frac{EI(O_t - EO - EOI)}{EOI} \quad (12)$$

2. I = Fatty acid amides

Fatty acid amides, unlike the free acids, were found to form ternary complexes ERI, as indicated by changes in fluorescence intensity (See Table 4)

and by decreased values for the apparent $K_{E,R}$. Binary complexes EI (and ER) are also formed. The mode of calculation is somewhat different in cases where Q_{ERI} differs markedly from Q_{ER}, and in cases where Q_{ERI} is $\approx Q_{ER}$.

$$Q_{ERI} \neq Q_{ER}.$$

In the presence of large excess of I, E and ER are negligible. Titration with R therefore gives the value of $K_{EI,R}$. In addition one obtains the Q-values for ERI (Q_{ERI}) which varies for different amides (See Table 4). In the cases where Q_{ERI} is sufficiently different from Q_{ER} (≈ 13.5), as for example with *iso*butyramide, this offers a possibility of determining the ratio ER/ERI experimentally at intermediate I. When $I >> EI + ERI$, which is mostly the case

$$K_{ER,I} = \frac{ER \times I}{ERI} \tag{13}$$

and the ratio ER/ERI is independent of R and remains constant through the titration, just as the fluorescence increase factor Q_{ER+ERI} for the mixture of ER + ERI. Therefore

$$\frac{ER}{ERI} = \frac{Q_{ERI} - Q_{(ERI+ER)}}{Q_{(ERI+ER)} - Q_{ER}} \tag{14}$$

On the other hand, ER + ERI can be calculated using Q_{ER+ERI} for any point of the titration curve. These values of ER/ERI and ER + ERI give ER and ERI which if inserted in either of the equations

$$K_{EI,R} = \frac{EI(R_t - ER - ERI)}{ERI} \tag{15}$$

or

$$K_{E,R} = \frac{(E_t - EI - ER - ERI)(R_t - ER - ERI)}{ER} \tag{16}$$

give EI, and allow the calculation of $K_{E,I}$.

If now another titration with R is carried out on a solution containing the same amounts of E and I, but in addition a suitable amount of O ($[DPN] \approx K_{E,O}$) it is found that the calculated values of EI, ER and ERI when introduced into (16) give a higher value than $K_{E,R}$ ($= K_{E,R,app}$). This was to be expected since some of the free E is bound to O, but it could not be predicted whether this occurs both as EO and EOI. It was found, however, that under the assumption that *only* EO is formed, the values for EO, calculated from

$$K_{E,R} = \frac{(E_t - EI - ER - ERI - EO)(R_t - ER - ERI)}{ER} \tag{17}$$

and inserted into

$$K_{E,O} = \frac{(E_t - EI - ER - ERI - EO)(O - EO)}{EO} \tag{18}$$

gave values for $K_{E,O}$ which agreed as well as could be expected with the values for $K_{E,O}$ obtained in titrations without inhibitors; see Table 5. *The conclusion is that EO, unlike ER, does not form ternary complexes with fatty acid amides.*

$$Q_{ERI} \approx Q_{ER}.$$

In this case the experiments give values only for $ER' = ER + ERI$. $K_{EI,R}$ is determined directly in experiments with large excess of I. Then from experiments with intermediate I the ratio EI/ERI is obtained from

$$\frac{EI}{ERI} = \frac{K_{EI,R}}{R_t - ER'} \tag{19}$$

which together with the equations

$$ER' = ER + ERI \text{ and} \tag{20}$$

$$K_{ER} = \frac{(E_t - EI - ER')(R_t - ER')}{ER} \tag{21}$$

allows the calculation of the three unknown ER, EI and ERI, and the corresponding dissociation constants. Experiments in the presence of DPN are evaluated as described above.

In the case of high affinity of the inhibitors for the binary complexes, sometimes such small concentrations of I had to be used that corrections for the amount of bound I had to be introduced.

As is easily derived from the above formulae the dissociation constants for the binary and ternary complexes are related by

$$\frac{K_{EI,R}}{K_{E,R}} = \frac{K_{ER,I}}{K_{E,I}} = \frac{EI \times ER}{ERI \times E}, \tag{22}$$

and *vice versa* for the complexes with DPN. When the constants are calculated as described above, eqn. 22 serves as an excellent check on the correctness of the calculations. The data given in Tables 1, 2, 3 and 5 have all been found to fulfil condition (22) within the limits of error.

Table 1. The effect of saturated fatty acids on the dissociation constants at pH 7 23.5°C ($K_{E,O} = 84$).

Chain Length	$K_{EI,O}$	in μM $K_{EO,I}$	$K_{E,I}$
C_1	5.2	2 900	47 000
C_2	7.2	9 500	100 000
C_3	5.7	2 700	40 000
C_4	2.1	1 600	63 000
C_5	4.1	1 400	35 000
C_6	7.0	300	3 100
C_7	2.5	131	4 000
C_8	6.6	84	1 100
C_9	4.2	40	700
C_{10}	1.8	5.0	230
C_{11}	11.0	1.4	10.2
C_{12}	6.4	0.8	10.3
C_{13}	38.0	4.0	9.9
C_{14}	35.0	3.8	9.1
C_{15}	11.0	0.7	5.7
C_{16}		24	$>>58$
C_{17}		22	$>>22$
C_{18}		26	$>>21$
C_{19}		28	$>>18$
C_{20}		22	$>>20$

RESULTS

The dissociation constants $K_{E,I}$, $K_{EI,O}$ and $K_{EO,I}$ (I = Fatty acid)

It is seen from Table 1 that fatty acids exhibit strong ternary complex formation with ADH · DPN complex and that the affinity on the whole increases strongly with the length of the carbon chain of the acid up to C_{15}. The dissociation constant for the ternary complex ADH · DPN · fatty acid, $K_{EO,I}$, decreases from 2 900 μM for formate and 9 500 for acetate to 0.7 μM for C_{15} acid, while the dissociation constant for fatty acid and ADH, $K_{E,I}$, decreases from 47 000 μM for formate and 100 000 for acetate to 6 μM for C_{15} fatty acid. The dissociation constant reflecting the effect of DPN on the ADH · fatty acid complex, $K_{EI,O}$, is rather constant at about 5 μM to the C_{11} acid, and then increases from 2—7 times this value from C_{11}—C_{15}. The ability of the fatty acids to form complexes and the dissociation constant reflecting the formation of a ternary complex between EO and I, $K_{EO,I}$, increasing to a constant level of about 25 μM for the acids C_{16} to C_{20}.

Table 2. Dissociation constants (K) for complexes of liver ADH (E) with DPN (O), DPNH (R) and capric acid (I). 23.5° C, phosphate μ = 0.1.

	pH 6	pH 7	pH 8
$K_{E,R}$	0.26 μM	0.38 μM	0.45 μM
$K_{E,O}$	107 »	84 »	28 »
$K_{E,I}$	31 »	200 »	290 »
$K_{EO,I}$	1.1 »	3.8 »	11.4 »
$K_{EI,O}$	3.7 »	1.6 »	1.1 »

Table 2 shows the effects of capric acid (C_{10}) at different pH-values. The dissociation constants $K_{E,R}$ and $K_{E,O}$ were taken from independent determinations with the same enzyme solution and used to calculate the other constants. It can be seen that both $K_{E,I}$ and $K_{EO,I}$, the constants reflecting the effect on inhibitor on free ADH and on ADH · DPN complex, increase with increasing pH while the constant reflecting the effect of DPN on ADH · inhibitor complex decreases as the pH increases. The presence of fatty acid stabilizes the binding of DPN in the ternary complex, ADH · DPN · fatty acid, particularly at pH 6, and *vice versa*, DPN stabilizes the fatty acid in the ternary complex.

The dissociation constants $K_{I,E}$, $K_{EI,R}$ and $K_{ER,I}$ of fatty acid amide complexes.

Table 3 shows the effects of amides as indicated by the various dissociation constants. The presence of inhibitor lowers the dissociation constant $K_{EI,R}$ from 0.31 to 0.02 μM for the amides C_4—C_6 whereas it is slightly higher for the lower amides. The lowering of the constants, reflecting the effects of inhibitor on free ADH and on ADH · DPNH complex is about 10 000 times from acetamide to hexamide. Column 6 in Table 3 is the calculated $K_{E,O}$ as determined

Table 3. The effect of straight chain amides on the dissociation constants at pH 7, 23.5°C ($K_{E,R} = 0.31$).

Chain Length	$K_{EI,R}$	in μM $K_{ER,I}$	$K_{E,I}$	[DPN]	$K_{E,O}$ (calc.)
C_1	0.11	103 000	360 000	193	84
C_2	0.05	5 000	36 000	306	88
C_3	0.025	505	6 170	109	125
C_4	0.020	64	1 000	218	130
C_5	0.020	25	400	109	89
C_6	0.020	11	165	218	95

from eqn. 15. The amount of DPN used to determine the constants is listed in column 5 of the table. As stated in the experimental section, the determination of the Q_{ERI}-values for the various amides used to determine the constants was made by one of two methods. Table 4 states the values of Q_{ERI} for the amides tested together with the concentration used and the method of determination. The Q_{ERI}-values increase from formamide to propionamide and then decrease from butyramide to hexamide. The value for *iso*butyramide is specially high, about 3 times the value obtained in the absence of inhibitor. Q is independent of pH from 6—9. The fluorescence changes observed with acetamide are shown in Fig. 1. As previously noted [7,8], the shift of wavelength observed when DPNH combines with ADH is about 20 mμ from 472 mμ to 450 mμ (uncorr.). The further shift to 435 mμ is observed in the presence of nearly saturating amounts of acetamide. A similar shift to wave length when the ADH · DPNH ·

Table 4. Q_{ERI} values for amides at pH 7. Emission recorded at 410 mμ. $Q_{ER} = 13.5$ (in absence of inhibitor).

Amide	Concentration mM	Method of determination	Q_{ERI}
Formamide	250	Calculated	11.7
Acetamide	250	Directly	16.5
	10	Calculated	16.6
Propionamide	6.1	Directly	18.9
	30.4	Directly	19.0
	30.4	Calculated	20.3
	3.04	Calculated	18.4
Butyramide	50.5	Directly	15.6
	50.5	Calculated	13.4
	5.05	Calculated	14.5
*Iso*butyramide	40.7	Directly	40
Valeramide	0.244	Calculated	8.9
	0.122	Calculated	8.4
*Iso*valeramide	19.3	Directly	11.7
	0.384	Calculated	11.8
Hexamide	0.0259	Calculated	11.1

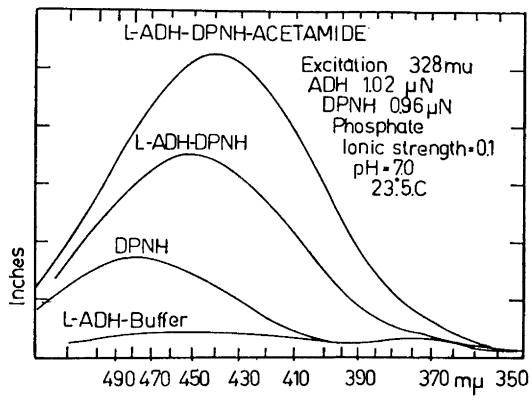

Fig. 1. The effect of adding acetamide to a partly dissociated L-ADH-DPNH complex; the fluorescence increases both because a much less dissociated ternary complex is formed and because the ternary complex has a somewhat higher Q-value than for ER.

amide complex was formed is seen with all amides tested, independent of the fact noted in Table 4 that some amides actually quench, others increase, to varying extents, the fluorescence of ADH · DPNH. Ternary complex formation with ADH—DPNH is specific for carbon chain amides and is not observed with glutamine, nicotinamide or succinimide. Benzamide, if analogous to benzaldehyde (which is a good substrate for liver ADH [4]), would form a ternary complex with ADH—DPNH, but such a complex, if formed, is obscured by the non-specific quenching of this amide. Partly dissociated complexes of lactic and glutamic acid dehydrogenase of the type ER show no observable fluorescence changes at either the protein or DPNH wave lengths upon the addition of acetamide.

Table 5 indicates the changes in the dissociation constants with pH. The constant $K_{E,R}$ was determined from independent experiments without amides, and $K_{E,O}$ was calculated using eqn. 15 from titrations of ADH + DPN + ami-

Table 5. Dissociation constants (K) in μM for complexes of liver ADH (E) with DPN (O); DPNH (R) and *iso*butyramide (I) at 23.5°C.

	pH 6	pH 7	pH 8	pH 9
$K_{E,R}$	0.25	0.31	0.45	0.95
$K_{E,O}$	125	88	22	15
$K_{E,I}$	270	5 750	930	540
$K_{ER,I}$	180	450	210	120
$K_{EI,R}$	0.17	0.02	0.10	0.20

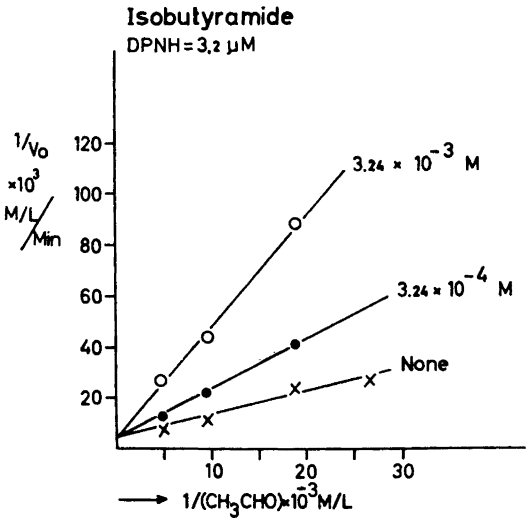

Fig. 2. Lineweaver-Burk plots demonstrating the competitiveness of *iso*butyramide with acetaldehyde. pH = 7.0. Phosphate buffer ionic strength 0.1; temp. 23.5°.

des with DPNH. The agreement of these values for $K_{E,O}$ with the earlier values[3] give strong support to the assumption that ternary complexes between ADH, DPN and amides are not formed.

Competition of amides and fatty acids for the aldehyde and alcohol binding sites respectively.

As noted in the introduction formate was earlier found from reaction velocity constant measurements to compete with ethanol. Fig. 2 shows a typical Lineweaver-Burk plot of the competitiveness of *iso*butyramide with acetaldehyde.

Fluorescence emission data of amide and fatty acid complexes when excited with 289 mμ light.

As Velick [10] has reported with glyceraldehyde phosphate and heart muscle lactic dehydrogenase, the protein fluorescence at 350 mμ when activated at 289 mμ is quenched by either DPN or DPNH. Fig. 3 shows a typical spectrum of the effects observed at 350 mμ when DPNH in a final concentration of 0.70 μM is added to an 0.59 μN ADH solution in phosphate buffer, pH 7. The fluorescence of ADH is quenched somewhat but there is no shift in the wave length when ADH · DPNH complex is formed. The addition of 2.6×10^{-2} M acetamide quenches the fluorescence and shifts the wave length about 10 mμ to 360 mμ. The emission bands in the visible wave lengths when ADH · DPNH and ADH · DPNH · acetamide complexes are formed are similar to those

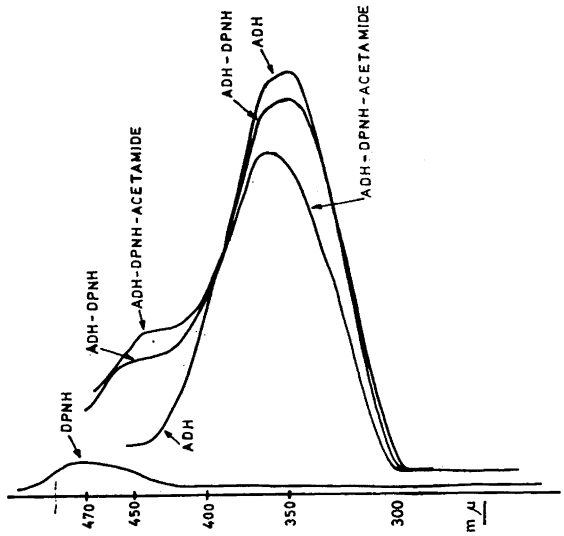

Fig. 3. Emission spectra of ADH, ADH—DPNH and ADH—DPNH-acetamide, when activated by 289 mμ. Complex formation with DPNH causes slight quenching and shift of maximum emission from 350 to 360 mμ. The emission of the complexes in the visible light is presumably caused by energy transfer from the protein moiety to the coenzyme.

shown in Fig. 1. No effect is seen when fatty acids are added to the ADH·DPNH complex nor is any effect observable when an amide is added to the ADH · DPN complex. The latter observations support the conclusion that ternary complexes are not formed in these cases.

DISCUSSION

While the measurements of dissociation constants by the fluorometric technique indicates the existence of complexes of the type ADH · DPNH · fatty acid and ADH · DPNH · amide, it is not immediately apparent that these complexes bear any relation to the enzymatically active complexes. However, the following lines of evidence suggest that such complexes are indeed similar in structure to the activated complexes involved in the interconversion of ADH, DPNH, aldehyde and ADH, DPN, alcohol:

1. The fluorometric results can be fitted to a mathematical treatment which is based upon the postulate that in the case of amides no EOI or OI and in the case of fatty acids no ERI or RI is formed.

2. Fatty acids show competitive behaviour with ethanol, and fatty acid amides with acetaldehyde.

3. The spectroscopic emission data of the amide complexes both at the pyridine nucleotide fluorescence band and at the protein fluorescence band and of the fatty acid complexes at the protein fluorescence band indicate the analogy with true enzyme-coenzyme-substrate complexes.

4. The inhibition effect of acids and amides increase with the length of the C-chain just like the enzymatic activity on alcohols and aldehydes, as indicated from substrate specificity studies.

5. Neither amides nor fatty acids show any observable effects with other pyridine-coenzyme dehydrogenase complexes studied, such as lactic acid dehydrogenase-DPNH and glutamic acid dehydrogenase-DPNH.

6. The observation of ternary complex formation of lactic dehydrogenase · DPNH with anions such as oxamate and oxalate has recently been reported by Winer and Schwert [5]. The values of the dissociation constant of oxamate from the LDH · DPNH · oxamate complex were found to be of the same order of magnitude, and to vary in the same way with pH, irrespective of whether the values were estimated from kinetic inhibition or from quenching experiments.

The effect of saturated fatty acids and fatty acid amides in lowering the dissociation constants indicates that the substrate analogs stabilize the binding of the coenzyme with ADH. *Vice versa*, the coenzyme stabilizes the binding of I on the ternary complexes. In the case of the acids the relative magnitudes of $K_{EI,O}$ and $K_{EO,I}$ indicate that from C_1 to C_{10} I dissociates easier than O from EOI, whereas the reverse is true from C_{11} to C_{15}. The *preferred* * order of the dissociation reaction of EOI is thus from C_1 to C_{10} the same as that assumed for ADH—DPN-alcohol by Theorell and Chance [13], but is reversed for C_{11} to C_{15}. In the case of acetate and formate it appears, as had been indicated earlier from kinetic data [1], that acetate dissociates easier from ADH and from ADH—DPN than does formate. It should be pointed out that the apparently irregular variations in the dissociation constants with stepwise increasing chain length may be largely due to experimental errors. The general trend is, however, very obvious.

The low solubility in water of the potassium salts of the acids from C_{16} upwards prevented the use of higher concentrations, but it is clear from the results that complexes of the type EI for C_{16} to C_{20}, if existent, are much more dissociated than C_{11} to C_{15}, see Table 1. The sudden change between C_{15} and C_{16} may reasonably be attributed to difference in micelle formation, and would in such case be expected to be temperature dependent. Further investigations are needed to solve this problem.

It is evident from Table 2 that changes in pH can invoke changes in the preferred order of reaction, as seen from a comparison between the values for $K_{EO,I}$ and $K_{EI,O}$. At pH 8 I is much more easily dissociated from EOI than is O, as postulated by Theorell and Chance [13] for a ternary ADH · DPN · ethanol complex. But at pH 6 the order is reversed, so that O is more easily dissociated from EOI than is I. This could explain the discrepancies between the values for $K_{E,O}$ found in equilibrium and kinetic measurements [3]. The kinetic values would then be calculated on the basis of a wrong assumption. The differences were by far the greatest in acid solutions, as we indeed would expect if a qualitative analogy can be drawn between the EOI and EO-ethanol complexes. Too high values for $K_{E,O}$ would be found in kinetic experiments in case the presence

* We consider *"preferred"* order rather than *"compulsory"* order more appropriate when speaking of enzyme-coenzyme complexes since the order may change depending on the experimental conditions employed. This has already been pointed out by one of us (H.T.) [12].

of ethanol in the ternary complex would loosen the attachment of DPN, thus increasing the "off" velocity rate constant *.

In the case of *iso*butyramide (see Table 5), $K_{ER,I}$ is $\gg K_{EI,R}$ at all pH values. This indicates that the dissociation of the ADH · DPNH · amide complex preferably leads to ER + I, whereupon the dissociation of ER goes much easier. If we assume, by analogy, that $K_{ER,S}$ is $\gg K_{ES,R}$, where S means acetaldehyde, the dissociation velocity constant (k_2) of ER would most probably be rate limiting in the reaction between ADH and an excess of DPN and alcohol, since the large difference between $K_{ER,S}$ and $K_{ES,R}$ could scarcely depend only on differences between the association velocity constants of ER to S and ES to R. The kinetic experiments should therefore be expected to give the true values of $k_2 \cdot K_{E,R}$ as determined either in equilibrium or in kinetic measurements agreed at all pH values [3], that is $K_{E,R} = k_2/k_1$ (where k_2 is the dissociation, k_1 the association velocity constant). Since $K_{E,R}$ and k_2 are most probably essentially correct we may draw the conclusion that the value found for k_1 in kinetic experiments must be near the true one too. Why is this so? $K_{E,I}$ for *iso*butyramide is certainly much larger than $K_{E,R}$ (See Table 5), which could depend in part on the association velocity constant of E + R being larger than that of E + I. If, by analogy, the same holds true for E + R *versus* E + S (aldehyde) this would imply preferred formation of ER in a mixture E + R + S. However, in the kinetic experiments k_1 becomes rate-limiting only at low [R] and high [S], so that E will nevertheless perhaps combine with S before it combines with R. If this is the case, the kinetic experiments can give true values for k_1, only if the association velocity constants of E + R and of ES + R are about the same.

The formation of ternary complexes with long-chain acids and amides is in line with the substrate specificity studies on the liver ADH system with different aldehydes and alcohols recently reported by one of us (A.D.W.) [4] **. Higher alcohols such as octanol and decanol are good substrates whereas cetyl- and stearyl-alcohols are very poor substrates, and both of the latter alcohols show about the same initial reaction velocities under identical experimental conditions ***. The failure of the fatty acids from C_{16} upwards to form complexes of the type EI may reflect the orientation of the fatty acid on the substrate site. If it is considered, therefore, that aldehydes and amides on one side, and alcohol and acids on the other side function as structural analogs, there is strong suggestion that the ADH · DPNH · amide and ADH · DPN · fatty acid complexes, in which no net reaction occurs, represent complexes analogous with those formed between ADH, DPNH, aldehyde and ADH, DPN · alcohol.

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* An example of ternary complex formation leading to $K_{EI,R}$ being $> K_{E,R}$ has recently been observed by H. Theorell and J. S. McKinleyMcKee (unpublished) in the highly fluorescent complex ADH · DPNH · imidazole.

** With yeast ADH, ternary complexes of the type ERI with amides and EOI with fatty acids are also formed. However, in this case increasing chain length does not seem to increase the stability of the complexes appreciably (unpublished observations of Thomas Langan Jr.). This is in accord with the very different specificity requirements of the yeast enzyme.

*** A manuscript describing the specificity of higher alcohols on the liver alcohol dehydrogenase system is in preparation.

Thus, from these inhibition studies of substrate analogs, it seems that not only is the coenzyme important for the binding of substrate but also the substrate is important for the binding of coenzyme. One should perhaps speak of enzyme-coenzyme specificity rather than enzyme specificity. In the natural enzymatic reaction involving a simultaneous change of one form of the coenzyme to another and a corresponding change in the substrate, it would appear that the change from one to another requires a coordinated change in molecular shape of both coenzyme and substrate. The fact that the substrate analogs were found to form ternary complexes with ADH and either, but not both, DPN or DPNH, suggests that the reactive ADH · DPN · alcohol and ADH · DPNH · aldehyde complexes can be formed, but not the non-reacting ADH · DPN · aldehyde or ADH · DPNH · alcohol complexes. Such complexes would involve a corresponding inhibition of enzyme activity and Nature has, it seems, found stereochemical means of preventing their formation at least to any disturbing extent.

Remark by H.T., February 10, 1960. The experimental work described here was finished in June 1959. Refinement of the fluorometric technique has afterwards revealed that DPN to some extent lowers the Q-values of ER. As a consequence hereof the values for $K_{E,O}$ given in this paper and in Ref.[14] (84—125 μM) are somewhat too low. A more probable value is 160 μM. This discrepancy does not affect the general conclusions, but the absolute values of the dissociation constants $K_{EO,I}$ and $K_{EI,O}$ may have to be somewhat revised.

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