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Liver Alcohol Dehydrogenase

II. Equilibrium Constants of Binary and Ternary Complexes of Enzyme, Coenzyme, and Caprate, *Iso*butyramide and Imidazole

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Using the fluorescence of the complexes ER and ERI the dissociation constants in 0.1 μ buffer, 23.5°C, at pH 7 of the caprate complexes $K_{E,I}$, $K_{EO,I}$ and $K_{EI,O}$ and of the *iso*butyramide complexes $K_{E,I}$, $K_{ER,I}$ and $K_{EI,R}$ have been determined, as well as $K_{E,R}$, $K_{E,O}$ and the imidazole dissociation constants $K_{E,I}$, $K_{ER,I}$, $K_{EI,R}$, $K_{EO,I}$ and $K_{EI,O}$ for the pH range 6—9. Glycine used for buffering at pH 9, was found to give a binary but no ternary complex. In titrations in the presence of relatively high concentrations of alcohol, "unnatural" binary and ternary alcohol complexes were also found.

$K_{E,R}$ was similar to previous values, but higher values obtained for $K_{E,O}$ resulted now in the disappearance of previous disagreements between the equilibrium and kinetic values at lower pH's, the results now agreeing with the Theorell-Chance mechanism.

Except for $K_{E,R}$, $K_{E,O}$ and $K_{EO,I}$ the dissociation constants were essentially invarient with pH. The increase of $K_{E,R}$ at high pH suggested it was probably sulphydryl groups which joined the protein to the DPNH ring. Analysis of the pH curve for $K_{E,O}$ indicated that of the free octahedral zinc bonds which in the free enzyme presumably hold water, one at higher pH (8.6) looses a proton. The resulting attraction between zinc-OH^- and the positively charged pyridine ring of DPN, is considered responsible for the changes in $K_{E,O}$ with pH.

In a previous publication, Winer and Theorell[1] described how fatty acids (I) formed complexes EI with liver alcohol dehydrogenase (E), as well as complexes EOI with the binary complex EO of enzyme with diphosphopyridine nucleotide, DPN (O). The corresponding complexes with DPNH, ERI, were not formed with fatty acids.

Fatty acid amides, on the contrary, formed complexes of the type ERI, but not EOI.

While testing different nitrogen compounds for their ability to form ternary complexes with ER it was observed that imidazole gave highly fluorescent compounds with interesting properties. Imidazole forms complexes with zinc

ions [2,3], and LADH contains zinc [4] just as does yeast ADH [5]. It was considered interesting to make more accurate determinations of the dissociation constants of the complexes of one fatty acid and one amide than had been possible previously. Capric acid and *iso*butyramide were selected, the latter because of its highly fluorescent ternary complexes, the former because it was found in determinations of $K_{E,O}$ that DPN not only competes with DPNH but in addition causes some quenching of the fluorescence intensity of ER. Thus a redetermination of the dissociation constants of the ternary complexes of caprate with DPN was called for, as the relations between these constants are so involved that even small errors sometimes produce unexpectedly large effects. In addition, the imidazole complexes were studied, with the purpose of comparing all these equilibrium data with studies of the influence of these substances on the kinetics.

EXPERIMENTAL

Buffers. At pH 6—8, 0.1 μ phosphate buffer was used while at pH 9, (a) 0.1 μ phosphate, pH 8 + 0.1 NaOH-glycine buffer, pH 10, gave 0.1 μ phosphate + 3.64 mM glycine buffer. (b) 1 M NaOH + glycine crystals gave 0.1 μ NaOH + 811 mM glycine buffer and (c) a mixture of the above two gave a 0.1 μ phosphate + 81 mM glycine buffer. Versene was never present in these or any other solutions.

Enzyme and coenzymes. LADH prepared according to Dalziel [6] or Bonnichsen and Brink [7] was used. The two enzymes gave identical results. In step 5 the Bonnichsen enzyme was twice dissolved in ice cold 0.02 M phosphate pH 7, and precipitated with 30 % ethanol. The crystals were each time washed with a little buffer containing 6 % ethanol. The enzyme was then crystallized from 0.02 M phosphate, pH 7, at 2°C. Finally it was recrystallized several times by dialysis [6]. The enzyme was considered pure, the absorbancy index at 280 mμ being 35×10^4 cm^2/mole (specific extinction = 0.42)[8], while E_{280}/E_{260} which was independent of whether the enzyme was treated with DPN (see below) or not, was 1.30 ± 0.01. A suspension of LADH crystals in 0.1 μ phosphate and 30 % alcohol (~ 10 mg/ml) was centrifuged; the crystals were dissolved in the same volume of 0.1 μ phosphate-ammonia buffer, pH 9, centrifuged again and dialysed twice against 0.1 μ phosphate, pH 7 or 8. A few milligrams of DPN/ml were then added to the enzyme which was then kept overnight in the refrigerator. The enzyme solution was then dialysed 4—5 times (3—5 days) against phosphate buffer. The DPN treatment was made because it is essential for good titrations in the presence of DPN that the enzyme does not contain traces of alcohol [9]. If precautions are taken to ensure that the quartz distilled water (distilled water redistilled from dilute alkaline permanganate) used to make up the buffer solutions and the buffer solutions themselves never are near a room where there are traces of alcohol in the air the DPN treatment can be avoided and contrary to previous ideas [9] alcohol can be removed by dialysis alone, the resulting enzyme giving identical results with the DPN treated enzyme. The above conditions are indeed important in these experiments (as a problem is that the enzyme quickly picks up any traces of alcohol in the air). Stock enzyme solutions $0.5—0.6 \times 10^{-4}$ M resulted which were quite stable, their activity decreasing approximately 1 % each day. Enzyme assay was carried out by determining the concentration of binding sites (1.0—1.2 μN in most equilibrium experiments) through titration with DPNH in the presence of 50 mM *iso*butyramide. This was performed at the beginning and end of each day's experiments, there normally being no change. The details regarding the coenzymes were described in Part I.

Inhibitors. Potassium caprate and *iso*butyramide did not fluoresce. Most nitrogen compounds, however, like imidazole were found to fluoresce strongly due to impurities. As in both kinetic and equilibrium experiments this background fluorescence prevents the use of high apparatus sensitivity, it was necessary to prepare the nitrogen compounds as fluorescence free as possible. This was achieved by adsorbing the fluorescent impurities on Norite S X 30 charcoal. To avoid contamination with organic solvents such as ethanol from which Tris is usually recrystallized or benzene (F = 2.6, S = 1, see Table 1) from

Table 1. Fluorescence F in inches at sensitivity $S = 1$. Water = 0.1″.

Compound	Tris	Imidazole	Piperazine
2 M Solution	2.2	27.0	7.4
1st Recrystallisation	0.9		
2nd »		2.5	0.7
Norite Column	0.2	0.35	0.2

which imidazole is usually recrystallized, the nitrogen compounds even when very soluble were recrystallized from water at temperatures down to 0°C. A 2 M solution in water was then passed once or twice using a 30 cm mercury pressure, through a fresh 3—4 cm column of Norite S X 30, previously washed with 2 N HCl, 2 N ammonia and water. This treatment reduced the fluorescence of imidazole and the nitrogen compounds used to near that of water (Table 1). The process was accelerated if the 2 M solution was first heated with Norite, filtered hot and then allowed to crystallize. Water was removed around 40°C and the fluorescence-free compounds dried and kept as crystals. The liquid nitrogen compounds tried, were soluble in water and were passed directly through Norite-water columns as above.

As it was necessary to have an accurate pK for imidazole under our experimental conditions, so as to know the concentration of neutral or unionized imidazole, 0.2 M imidazole was titrated with 1 M HCl at 23.5°C, using the Beckman Model G pH meter used throughout our work. The pK of imidazole was taken as 7.10 as the titration gave 7.12 while the values in the literature when adjusted to 0.1 μ, 23.5° were 7.06[2], 7.10,[10] and 7.11 [11].

Experiments were performed with a recording Beckman spectrophotometer, DK2, to see if any addition compounds were formed between imidazole and the coenzymes under any of our conditions. None were formed.

Apparatus. The equilibrium experiments were performed using a recording spectrophotofluorimeter [12]. The activating wavelengths in light from a high pressure Xenon lamp were isolated using a Bausch and Lomb grating monochromator, passed into a 3 ml, 1 × 1 cm precision quartz cuvette containing either the experimental solution or the standard solution, and the emitted fluorescence analysed in a Beckman DU spectrophotometer with recorder. The quartz cuvette was placed in a light tight cell compartment maintained by water rapidly circulated from a thermostat at 23.5°C. This was the temperature of the experiments, the pH determinations (performed on each cuvette immediately after titration) and all solutions other than the concentrated enzyme and coenzyme solutions which were kept at 0°C. The standard (10.5 μM DPNH in 0.01 M Tris) was measured immediately before and after each experimental cuvette to ensure there had been no apparatus drift. In determining the dissociation constants, the activation wavelength was 330 mμ, the isosbestic point for DPNH and DPNH-LADH, while the fluorescence was measured at 410 mμ where the fluorescent ratio of DPNH-LADH/DPNH (Q) is 13.1. Using calibrated Carlsberg pipettes (3—200 μl), enzyme, coenzymes and inhibitor solutions were added either directly or on a glass rod to give a final cuvette volume of 2 ml.

METHOD OF CALCULATION

For all the inhibitors $K_{ER,I}$ was determined directly by titration of E in the presence of excess R with the inhibitor. (The ternary complexes comprising E, R and I can be written ERI or EIR. However, $K_{ER,I}$ represents I dissociating from the complex while $K_{EI,R}$ represents R dissociating from the same complex. The same terminology applies to the dissociation constants for the ternary complexes comprising O). Otherwise the constants were determined

from titrations with R. These gave a Q and an apparent dissociation constant D_{app} from which the true dissociation constants were calculated depending on how the titrations were set up and the fact that only the complexes ER and ERI fluoresce [1].

I. Titration of E with R. ($K_{E,R}$)

The cuvette only contains buffer and enzyme and as in all these titrations eight additions of R are made on a glass rod. Table 2 illustrates a typical calculation of Q and D_{app}. C (μN) = LADH concentration; R (column 1) = μM DPNH/addition; F (column 3) = $Q' - 1$, where Q' = deflection in inches for each addition of R/r; r = deflection that each addition of R would by itself give = (S—B) × 0.525/10.5; (S—B) = the deflection of 10.5 μM DPNH, S and B (see Figs. 4—5) being the deflection of the 10.5 μM standard and the buffer.

The eight additions of R have to be listed with the F values calculated for each resultant fluorescence increment. Column 2 is the summation of R and column 4 that for FR. Z (column 5) is the solution of the equation [12]

$$C\left(\frac{\Sigma R''}{\Sigma FR''} - \frac{\Sigma R'}{\Sigma FR'}\right)Z^2 - (\Sigma R'' - \Sigma R')\,Z + (\Sigma FR'' - \Sigma FR') = 0$$

for the particular pairs of ΣR and ΣFR values 1—5, 2—6, 3—7, 4—8. $Q = Z + 1$, an average of the four Z values being taken.

Our calculations were greatly simplified throughout by the use of a Wegematic 1 000 electronic calculating machine. Table 2 is typical of those it produced, as it had been programmed so that it was only necessary to feed in C and the columns R and F.

II. Titration of E + O with R ($K_{E,O}$)

In the titration of the enzyme with R in the presence of O, there is present in the cuvette at any stage E, ER and EO. If E_t, R_t, and E_f, R_f, respectively, represent total added or free E or R present, then

$$K_{E,R} = E_f \times R_f/ER = (E_t - ER - EO)(R_t - ER)/ER \qquad (1)$$

and $D_{app} = (E_t - ER)\,(R_t - ER)/ER$. D_{app}, the apparent dissociation constant, is the value given by the computor in the last column, see Table 2. Only when no other complexes than ER are present does this value represent a simple dissociation constant, $K_{E,R}$.

Putting $E_t = 1.0$ and ER = 0.5, EO is obtained from eqn. 2.

$$K_{E,R}/D_{app} = (0.5 - EO)/0.5 \qquad (2)$$

$E_f = E_t - ER - EO = 0.5 - EO$ and $K_{E,O} = E_f[O]/EO$.

Table 2. $K_{E,R}$ (0.1 μ, pH 7, 23.5°C). Expt. F IV, $C = 0.890$.

	R	ΣR	F	ΣFR	Z	ΣFR/Z	$K_{E,R}$
1	0.525	0.525	8.030	4.216	12.482	0.336	0.311
2	0.525	1.050	5.090	6.888	12.484	0.549	0.311
3	0.525	1.575	2.670	8.290	12.565	0.661	0.317
4	0.525	2.100	1.380	9.014	12.633	0.719	0.329
5	0.525	2.625	1.000	9.539		0.761	0.317
6	0.525	3.150	0.570	9.838		0.785	0.318
7	0.525	3.675	0.430	10.064		0.803	0.313
8	0.525	4.200	0.280	10.211		0.814	0.315
				Average	12.541		0.316

III. Titration of E + I with R ($K_{ER,I}$, $K_{E,I}$ and $K_{EI,R}$)

Whenever ERI is not formed and the only fluorescent complex is therefore ER eqn. 2 applies. If instead of O, it is I which is present, giving EI, then $K_{E,I}$ instead of $K_{E,O}$, results. This situation occurs with glycine and caprate.

When ERI can be formed, as with *iso*butyramide or imidazole, in titrations of E plus various amounts of I with R the complexes ER, EI and ERI are present. ER and ERI are fluorescent with different intensities, Q_{ER} and Q_{ERI}, and have different dissociation constants. Q and D_{app} are consequently found to vary with the concentration of I.

(a) Q_∞ and $K_{ER,I}$

From the variation of Q with [I] and knowing that at zero [I] $Q = Q_{ER}$, $Q_{ERI} = Q_\infty$ ([I] = ∞) can be caculated for pairs of Q values since

$$K_{ER,I} = \frac{ER}{ERI}[I] = \frac{Q_\infty - Q_x}{Q_x - Q_{ER}}[I] \quad (3)$$

and for any two concentrations of I

$$\frac{Q_\infty - Q_1}{Q_1 - Q_{ER}}[I_1] = \frac{Q_\infty - Q_2}{Q_2 - Q_{ER}}[I_2] \quad (4)$$

From the mean Q_∞, $K_{ER,I}$ is calculated directly using formula 3, or by plotting $\log \frac{Q_\infty - Q_x}{Q_x - Q_{ER}}$ against log [I], the value of log [I] at zero ordinate being log $K_{ER,I}$.

(b) D_{app}, $K_{E,I}$ and $K_{EI,R}$:

Put the total concentration of fluorescent complexes = ER′ = ER + ERI. Then by combining with (3):

$$ER = ER' \times K_{ER,I}/([I] + K_{ER,I}) \quad (5)$$

Knowing $K_{ER,I}$, and putting $E_t = 1.0$ and ER′ = 0.5, ER and ERI can be calculated for each concentration of I. Then

$$D_{app} = (E_t - ER')(R_t - ER')/ER' = (R_t - ER') = R_f \quad (6)$$

$$E_f = K_{E,R} \times ER/R_f \qquad (K_{E,R} = E_f \times R_f/ER) \tag{7}$$

$$EI = 0.5 - E_f \qquad (E_f = E_t - ER' - EI) \tag{8}$$

giving $K_{E,I} = E_f \times [I]/EI$ and $K_{EI,R} = EI \times R_f/ERI$

IV. Titration of E + I + O with R ($K_{EO,I}$ and $K_{EI,O}$)

Q and D_{app} depend on the various concentrations of both I and O. All the possible complexes may be present (ER, EO, EI, ERI, and EOI) but again only ER and ERI fluoresce. Eqn. 5 applies, giving ER and ERI which only depend on $K_{ER,I}$ and I.
Again

$$D_{app} = R_f \tag{6}$$

$$E_f = K_{E,R} \times ER/R_f \tag{7}$$

Now

$$EO = E_f \times [O]/K_{E,O}$$

$$EI = E_f \times [I]/K_{E,I}$$

$$EOI = E_t - (ER + ERI + EI + EO + E_f) = 0.5 - (EI + EO + E_f)$$

giving $K_{EO,I} = EO \times [I]/EOI$ and $K_{EI,O} = EI \times [O]/EOI$

Relations between the dissociation constants. All the dissociation constants are interdependent and have to fulfill relations 8 and 9

$$\frac{K_{ER,I}}{K_{E,I}} = \frac{K_{EI,R}}{K_{E,R}} \tag{8}$$

$$\frac{K_{EO,I}}{K_{E,I}} = \frac{K_{EI,O}}{K_{E,O}} \tag{9}$$

RESULTS

Table 3 gives the values at pH 6, 7, and 8 of K_{ER} at 23.5° and 0.1 ionic strength. As expected they do not differ very much from the earlier values [12]. The constancy of Q_{ER} both at these pH's and at pH 9 (Table 5) is noticeable and a mean $Q_{ER} = 13.1$ could have been used in these calculations. Indeed it is often best to use an average Q particularly when Q is dependent on small ER′ values as in the case of $K_{E\text{-capric}}$ in Table 8.

Table 4 gives the values for $K_{E,O}$ at pH 6—8. These have been calculated using the tabulated Q values from the computor, as in Fig. 1 the mean Q values obtained in $K_{E,O}$ determinations at pH 6—9 (Tables 4 and 6) in the presence of different amounts of DPN are plotted, and the resulting curve shows that there is a drop in Q with increasing concentration of DPN from 13.1 at zero DPN to around 11 at 1 mM, DPN obviously quenching the fluorescence somewhat. Though apparently not very large this decrease leads to considerably higher values for $K_{E,O}$, now for example at pH 7, 160 μM instead of 84 μM as calculated with an average Q previously [12]. In calculating D_{app} whenever DPN is present, each particular computor Q has always to be used.

Table 3. $K_{E,R}$ (μ = 0.1, 23.5°C)

pH	Expt.	Q	$K_{E,R}$ μM
6	F VI, E 3	13.70	0.225
	4	13.46	0.227
	F XA 12	12.66	0.226
		13.27 ± 0.26	0.226
7	F II, E 26	12.40	0.29
	III, 3 + 4	12.6	0.26
	IV, 5 + 6	13.94	0.317
	7 + 8 + 9	13.54	0.316
	F XA, 5	13.17	0.300
	6	13.31	0.367
	F XB, 3	12.43	0.359
		13.18 ± 0.17	0.310 ± 0.03
8	F VII, E 4	12.22	0.395
	5	12.67	0.425
	39	14.03	0.378
	46	13.65	0.433
	F XA 13	12.88	0.370
	14	14.32	0.467
		13.30 + 0.3	0.411 + 0.016

Glycine. At pH 9 glycine was used as buffering substance in earlier determinations [12]. As seen from Table 5 the values obtained for $K_{E,R}$ are strongly dependent on the glycine concentration. This was explained by assuming formation of an enzyme-glycine compound (EI) which does not combine with R or O. In agreement herewith Q is not at all dependent on the glycine concentration, and $K_{ER,app}$ is influenced to the same degree as $K_{EO,app}$. Calculations under this assumption with eqn. 2, gave $K_{E,glycine}$ = 230 mM. $K_{E,R}$ = 0.64 μM, and $K_{E,O}$ = 12 μM at zero [glycine] as shown in Table 7. Previous values for $K_{E,R}$ of 0.9—1.0 μM were all made in 100 mM glycine [12] and agree with the present results. The equilibrium and kinetic values can now be said to agree, not only for $K_{E,R}$ from pH 6—9 but also for $K_{E,O}$, the discrepancies for $K_{E,O}$ at neutral and acid pH previously [12] having disappeared. It was found practical to use in the kinetic experiments 3.6 mM glycine + phosphate. This small amount of glycine does not appreciably alter the kinetics but contributes to the buffering capacity which is very low for phosphate at pH 9.

Caprate. The dissociation constants $K_{E,I}$, $K_{EO,I}$ and $K_{EI,O}$ were redetermined for K-caprate. As seen from Table 8, $K_{E,I}$ now came out to be 45 ± 3 μM. This value is considerably lower than 200—250 μM obtained by Winer and Theorell [1]. It should be noted that the values for $K_{E,I}$ showed no drift with increasing concentration of caprate. This proves that a ternary ERI

Table 4. $K_{E,O}$ (0.1 μ, 23.5°C)

pH	Expt.	[DPN] μM	Q	D_{app} μM	$K_{E,O}$ μM
6	F VI, E 5	204	12.17	0.392	282
	6	408	12.13	0.573	266
	7	408	11.37	0.566	272
	8	1 020	10.0	1.166	246
	15	408	11.38	0.559	277
	16	408	10.95	0.595	250
	18	612	11.02	0.787	248
					266 ± 5
7	F III, E 5	136	11.7	0.495	191
	F IV, 10	136	13.16	0.635	135
	12	408	11.0	1.17	147
	14	408	11.0	1.05	171
	24	408	10.88	1.056	170
	25	408	11.23	0.995	165
	30	408	12.58	1.062	173
	26	816	11.39	1.957	157
	27	816	11.70	1.900	147
	28	816	11.10	1.55	187
	F XIV, 4	130	12.62	0.581	149
	11	128	13.40	0.584	144
	12	128	11.81	0.541	172
	13	320	10.66	1.008	143
					160 ± 4
8	F VII, E 9	68	13.30	1.134	38
	41	68	12.33	0.871	60
	43	68	13.64	1.052	43
	6	136	12.00	1.314	61
	7	136	12.41	1.414	55
					51 ± 5

Table 5. pH 9, $K_{E,R}$

Expt.	[Glycine] mM	Q	$K_{E,R}$ μM	$K_{E,R}$ average μM
F XII, 7	0	13.12	0.534	0.613
9	0	12.82	0.691	
F XII A, 10	3.6	13.08	0.714	
11	3.6	13.03	0.609	0.655
26	3.6	13.33	0.642	
F VIII, 6	81	13.66	0.884	
7	81	13.38	0.858	0.868
F IX, 23	81	12.66	0.891	
24	811	12.42	0.838	
F IX B, 44	811	14.12	2.94	2.63
46	811	13.02	2.33	
	Average	13.15		

Table 6. pH 9, $K_{E,O}$ in glycine-phosphate, 0.1 μ.

Expt.	[Glycine] mM	Q	D_{app}	[DPN] μM	$K_{E,O,app}$ μM
F XII, 12	3.6	10.66	2.663	34	11.1
13	3.6	11.26	1.689	17	11.1
F VIII, 8	81	11.88	4.172	68	17.9
9	81	12.78	4.75	68	15.2
10	81	13.93	4.13	34	18.0
11	81	12.81	1.82	17	15.4
F IX, 26	81	12.50	4.62	66	15.7
F IX B, 48	811	13.5	5.4	66	49
49	811	13.5	8.0	132	53
51	811	13.5	8.85	132	63

$K_{E,O}$, 3.6 mM: 11.1 ± 0 (2 values)
81 » : 16.4 ± 0.7 (5 values)
811 » : 55 ± 5 (3 values)

Table 7. pH 9, $K_{E,glycine}$ in glycine-phosphate, 0.1 μ.

[Glycine] mM	$K_{E,R,app}$ mM	$K_{E,O,app}$ mM	$K_{E,glycine}$ From $K_{E,R,app}$	$K_{E,glycine}$ From $K_{E,O}$	$K_{E,R}$ * [glycine]=0 μM	$K_{E,O}$ * [glycine]=0 μM
0	0.64 *	12 *			0.64	12
3.6	0.655	11.1			0.645	11.0
81	0.868	16.4	226	222	0.642	12.2
811	2.63	55	259	226	0.582	12.2
			Average 231 mM			

* Calc. values if $K_{E,glycine}$ = 231 mM.

Table 8. Caprate, $K_{E,I}$ (0.1 μ, pH 7, 23.5°C). Q_{ave} = 12.4.

Expt.	[Caprate] μM	D_{app} μM	$K_{E,I}$ μM
F XIV, E 15	50	0.59	55
5	100	0.89	53
6	100	1.19	35
7	100	1.10	39
8	200	1.68	45
9	200	1.59	49
10	400	2.90	48
11	400	3.32	41
		Average	45 ± 3

Table 9. Caprate, $K_{EI,O}$ and $K_{EO,I}$ (0.1 μ, pH 7, 23.5°C).

Expt.		[Caprate] μM	[DPN] μM	D_{app} μM	Q	$K_{EO,I}$ μM	$K_{EI,O}$ μM
F XVI, E	12	100	13.6	1.95	10.44	2.85	10
	13	100	6.8	1.60	11.24	2.25	6.9
	14	50	6.8	1.23	14.22	1.2	4.2
F XIV,	6	10	130	1.61	12.1	2.55	9.1
	8	5	65	0.96	12.1	1.3	4.6
				Average	12.0	2	7

complex is not formed with caprate, for in such case with increasing caprate D_{app} would asymptotically approach a constant value $= K_{EI,R}$. In agreement herewith the caprate caused no change in the Q-value.

The experiments for determining $K_{EI,O}$ and $K_{EO,I}$ which are interdependent through the relation $K_{E,O} \times K_{EO,I} = K_{E,I} \times K_{EI,O}$ (eqn. 9) are summarized in Table 9. In these experiments it was found difficult to get precise results, due to the great differences between $K_{E,O}$ and $K_{EI,O}$, $K_{E,I}$ and $K_{EO,I}$, respectively. Whatever the concentrations of DPN and caprate used, the complexes will be present in very different concentrations.

As seen from Table 9 a wide range of concentrations of both DPN and caprate were used. The values of the ternary complex constants are somewhat scattered, but entirely at random and the averages should not be far from correct. The values of Winer and Theorell, $K_{EO,I} = 3.8$, $K_{EI,O} = 1.6$ μM were calculated from $K_{E,O} = 84$ μM, $K_{E,I} = 200$ μM. If these determinations are recalculated using $K_{E,O} = 160$, $K_{E,I} = 45$ μM, we obtain: $K_{EO,I} = 1.7$ and $K_{EI,O} = 6.0$ μM, in agreement with the present determinations.

Iso*butyramide* (IB). Because of the strong fluorescence of the ternary ERI complex ($Q = 40$) the constant $K_{ER,I}$ could be determined by adding an

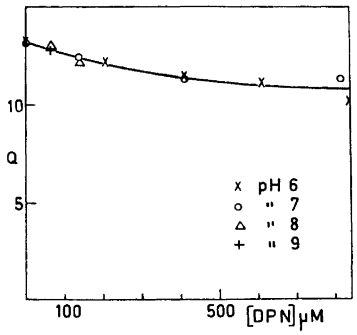

Fig. 1. Variation of Q with DPN concentration in $K_{E,O}$ determinations.

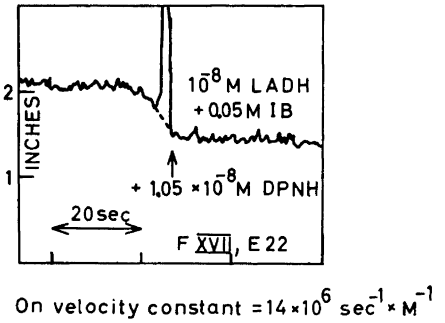

Fig. 2. Reaction cycle for E-*iso*butyramide associating with DPNH.

excess of R to E and then titrating with successive additions of either 10 or 50 μM IB. The results are given in Table 10.

The average value at pH 7, for $K_{ER,I}$ of 140 $\pm$ 5 μM, is definitely lower than the value 450 μM obtained earlier[1]. At pH 9, however, the earlier value $K_{ER,I}$ = 120 μM is in agreement with the present of 116 μM.

The influence of pH in changing from 7 to 9 is small, if it exists at all, for the values 116 and 115 at pH 9 were obtained in the same series and lie between the pH 7 values of 117 and 133 as can be seen from the experiment numbers.

Because of the very low value for $K_{EI,R}$, the direct titration of E + excess I with R implied working at extremely high dilutions. However, due to the high Q value we could when using highest sensitivity get deflections of around 2 inches when 10^{-8} M R was added to 2 $\times$ 10^{-8} N (10^{-8} M) E. The values for $K_{EI,R}$ (Table 10) now came out still lower than before, 5.5 $\times$ 10^{-9} M instead of 20 $\times$ 10^{-9} M.[1]

The value for $K_{E,I}$ was calculated from the values for $K_{E,R}$, $K_{ER,I}$ and $K_{EI,R}$. It was expected to be high; the earlier value was 5.75 mM. It now came out

*Table 10. Iso*butyramide. I. Direct titration of ER with 8 $\times$ 10 μM (or 50 μM in E 38—41) additions of *Iso*butyramide. μ = 0.1, 23.5°C.

pH	Expt.		[DPNH] μM	[LADH] μN	$K_{ER,I}$ μM
7	F XIV E	25	2.5	0.45	163
		26	5.0	0.45	152
		23	5.0	0.45	156
		29	10.0	0.90	135
		38	10.0	0.94	117
		41	10.0	0.94	133
	F XVI	16	10.5	0.85	145
		17	10.5	0.85	131
				Average	140 $\pm$ 5
9	F XIV	39	10.0	0.94	116
		40	10.0	0.94	115
				Average	116

II. Direct titration of EI with R ($K_{EI,R}$).

[LADH] = 2 $\times$ 10^{-8} N. [DPNH] Additions = 1.05 $\times$ 10^{-8} M. (0.1 μ, phosphate pH 7, 23.5°C).

Expt.		[*Iso*butyramide]	$K_{EI,R}$ M $\times$ 10^{9}	k_0 μM^{-1} $\times$ sec^{-1} [13] (from cycles)
F XVII	E 23	50 mM	6.0	13
	24	»	7.8	15
	25	»	4.0	15
F XVIII	27	»	4.6	
	29	»	4.5	
	33	»	6.0	
			5.5 $\pm$ 0.6	14

Table 11. Equilibrium constants (μM) for caprate and *iso*butyramide: 0.1 μ phosphate, 23.5°C, pH 7.

	$K_{E,I}$	$K_{ER,I}$	$K_{EI,R}$	$K_{EO,I}$	$K_{EI,O}$
Caprate	45	—	—	7	2
IB.	9 300	140	0.005	—	—

still higher, so that the 50 mM IB used in the experiment was not high enough to make corrections for free E in equilibrium with EI unnecessary. When these were done, $K_{E,I}$ was 9.3 mM, and the corrected $K_{EI,R} = 4.7 \times 10^{-9}$ M. This may be the lowest value for a dissociation constant ever determined by direct titration, illustrating the great sensitivity of fluorimetry.

An interesting observation was made in these titration experiments. At the high dilutions used the association of R with EI was slow enough to give measurable "on" cycles on the recorder (Fig. 2) from which the second order "on" velocity constant of R to EI could be calculated [13]. The value 14 $\mu M^{-1} \times sec^{-1}$ in Table 10 is close to the value of k_1 ($E + R \xrightarrow{k_1} ER$) = 11

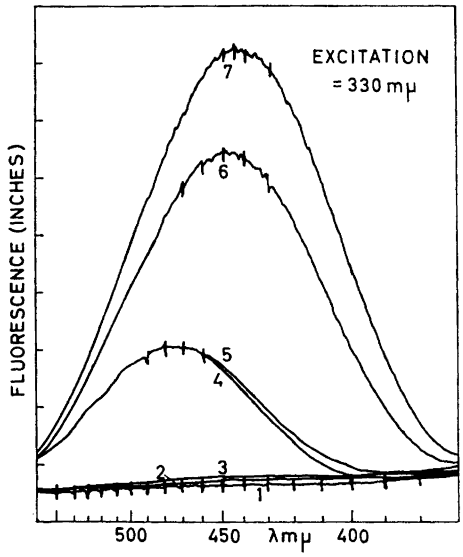

Fig. 3. Fluorescence emission curves for LADH, DPNH and imidazole.

1. Buffer (0.1 μ phosphate, pH7, 23.5°C)
2. » + 0.1 M imidazole
3. » + » + 2.2 μN LADH
4. » + 2.1 μM DPNH
5. » + » + 0.1 M imidazole
6. » + » + 2.2 μN LADH
7. » + » + » + 0.1 M imidazole

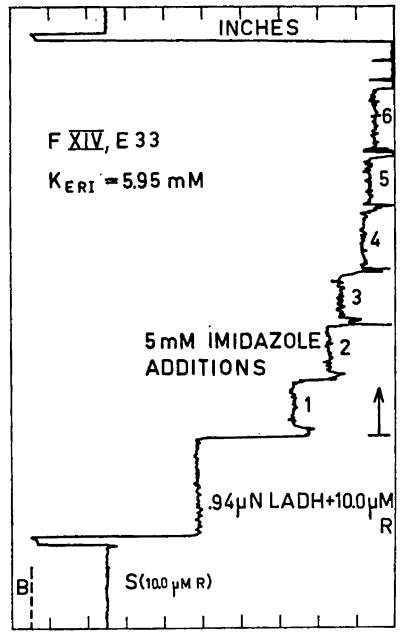

Fig. 4. Direct titration of LADH + DPNH with imidazole.

$\mu M^{-1} \times sec^{-1}$, obtained by kinetic experiments (see Part I). The great difference between $K_{EI,R} = 5 \times 10^{-9}$ M and $K_{E,R} = 310 \times 10^{-9}$ M must therefore be practically exclusively dependent on the "off" velocity constants. It should be emphasized that the association of DPNH to LADH is a very fast reaction of the same order of magnitude as the catalase reaction with hydrogen peroxide [14]. The association velocity constant of FMN to the apoprotein of the old yellow enzyme is under optimal conditions 1 $\mu M^{-1} \times sec^{-1}$.[13]

Imidazole. Fig. 3 shows the fluorescence emission curves for DPNH and its binary and tertiary complexes with enzyme and imidazole. The increase

Table 12. Imidazole; direct titration of $K_{ER,I}$, $\mu = 0.1$, 23.5°C, pH = 7.0.

Expt.		[DPNH] μM	[LADH] μN	$K_{ER,I}$ mM
F XIV, E	31	10	0.94	4.98 [a]
	33	10	0.94	5.93 [a]
	37	10	0.94	4.82 [b]
F XVI	18	10.5	0.85	5.5 [c]
	19	10.5	0.85	5.8 [c]
				5.41

a 5 mM additions
b 2 » »
c 2.5 » »

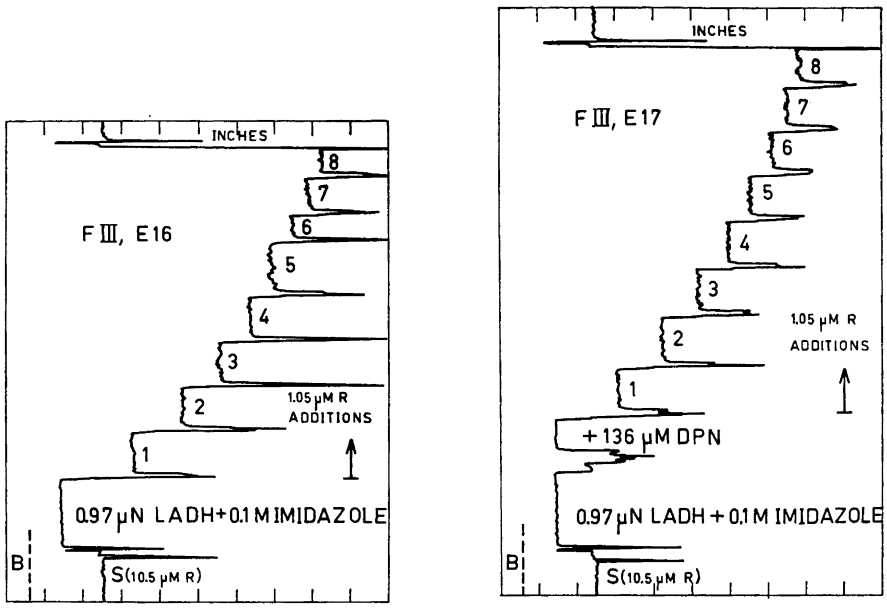

Fig. 5. (a) Titration of LADH + imidazole with DPNH. (b) Titration of LADH + imidazole + DPN with DPNH.

due to the formation of the ternary complex is particularly noticeable in view of the fact that although ERI is highly dissociated its fluorescence is still greater than that of the binary complex ER. The fluorescence emission curve for buffer, buffer with imidazole, or imidazole + enzyme could have been omitted as these are seen to be almost identical. The formation of the binary and ternary complexes is underlined by the change from 475—6 mμ (R) to 447 and 442 mμ in λ_{max} of their emission spectra. The maxima shown, however, are uncorrected for variations with wavelength of the intensity of the exciting light and photocell sensitivity and are 13 mμ too high, the true maxima being 462 (R), 432 (ER) and 429 (ERI) although the ternary complex being dissociated the latter value may be somewhat too high.

Fig. 4 shows a direct titration of E and excess R with imidazole at pH 7. Table 12 gives the values from these direct titrations, the mean $K_{ER,I}$ being 5.4 mM. This value is not too far from the value of 8 mM (Table 13) obtained as a mean of several determinations using the method of titrating E + various amounts of imidazole with R. The direct titration value for $K_{ER,I}$ might be expected to be most accurate as it does not involve Q_∞ which is sometimes difficult to obtain accurately. Fig. 5 shows two typical titrations with R, where compared with the titration of enzyme alone, imidazole markedly enhances the deflection per addition of R, and the total deflection, although these are both decreased again when O is present. In Figs. 4 and 5, B and S are the deflections of the buffer and the standard, the difference in the levels corres-

Table 13. Variation of Q and D_{app} with imidazole concentration at pH 7, 0.1 μ and 23.5°C.

Expt.	[Imidazole] mM	Q	D_{app}	$K_{ER,I}$(mM)	$K_{E,I}$(mM)	$K_{EI,R}$(mM)
FII, E 10	100	45.4	2.33	8.0	1.04	2.49
11	100	44.5	2.20	8.0	1.05	2.35
FIII, 16	100	43.8	2.12	8.0	1.05	2.26
18 + 20	100	44.3	2.12	8.0	1.05	2.26
		44.5				
FII, 20	30	38.4	1.51	8.0	1.35	1.83
21	30	38.0	1.59	8.0	1.28	1.93
22	30	39.9	1.63	8.0	1.25	1.98
FIII, 13 + 14	30	36.7	1.43	8.0	1.43	1.73
		37.9				
FII, 13	10	32.1	1.32	8.0	1.17	2.12
15	10	34.5	1.10	8.0	1.43	1.73
FIV, 16	10	35.7	1.29	8.0	1.19	2.08
20	10	33.6	1.19	8.0	1.31	1.90
		34.0				
FII 24	3	22.3	0.75	8.0	1.30	1.92
FIII 8	3	23.8	0.73	8.0	1.35	1.85
		23.1				
	∞	47.0	Average	8.0	1.23	2.03

ponding to the deflection of the DPNH in the standard solution, thus matching the apparatus sensitivity. Tables 13 and 14 summarize the results of the titrations with R at pH 7, while Table 13 also shows how markedly Q and D_{app} vary with imidazole concentration. Throughout all our experiments

Table 14. Variation of Q and D_{app} with imidazole concentration at pH 7, 0.1 μ and 23.5°C.

Expt.	[Imidazole] mM	[DPN] μM	Q	D_{app}	$K_{EO,I}$mM	$K_{EI,O}\mu$M
FII, E 12	100	136	43.0	2.54	3.06	393
FIII, 17 + 19	100	136	44.0	2.85	2.06	276
FII, 23	30	136	36.9	2.10	4.10	535
FIII, 15	30	136	36.8	2.11	4.11	535
FII, 16 + 18	10	136	31.8	1.71	3.46	452
FIII, 11	10	136	31.1	1.91	2.16	281
12	10	136	28.0	1.68	3.86	502
FIV, 18	10	136	33.1	1.72	3.40	443
17	10	408	31.2	2.46	4.20	545
22	10	408	29.7	2.42	4.20	545
23	10	816	27.0	3.87	3.68	478
FIII, 9	3	136	22.1	1.19	2.61	341
				Average	3.30	430

Table 15. Dissociation constants for complexes of LADH with the coenzymes and imidazole. $\mu = 0.1$ 23.5°C.

pH	Q	$K_{E,R}$ μM	$K_{ER,I}$ mM	$K_{ER,I}$ mM (Corr)*	$K_{E,I}$ mM	$K_{E,I}$ mM (Corr)*	$K_{EI,R}$ μM	$K_{E,O}$ mM	$K_{EO,I}$ mM	$K_{EO,I}$ mM (Corr)*	$K_{EI,O}$ μM
6	63	0.23	57	4.2	6.43	0.47	2.01	266	10.8	0.80	448
7	47	0.31	8	3.5	1.23	0.55	2.03	160	3.3	1.45	430
8	49	0.41	4	3.6	0.76	0.68	2.20	51	6.7	5.9	440
9	48	0.65	3	3.0	0.68	0.67	2.91	12	24.6	24.2	435
											440

* The total imidazole concentrations are corrected to the concentration of unionized imidazole using pK 7.10.

μ has been maintained constant at 0.1 except in some of these experiments at pH 7 where μ was > 0.1 due to the added contribution of imidazole-phosphate not having been allowed for. Consequently Q and D_{app} were corrected to $\mu = 0.1$ from plots of their variation with $\sqrt{\mu}$. It was found that a ten-fold increase in μ doubled D_{app} and the D_{app} values had to be reduced. Likewise with $\mu > 0.1$, Q was too low due to quenching by phosphate.

The mean values of the dissociation constants and their variation with pH, are given in Table 15. It is seen that the Q value of 63 is higher at pH 6 than at pH 7—9, where it is around 48. The reason for this is unknown. At pH 6 ER is a thousand fold more stable than EO, at pH 9 only twenty-fold, this fifty-fold change being caused by a 2- to 3-fold decrease in the stability of ER, and a twenty-fold increase in the stability of EO. $K_{ER,I}$ means that the total imidazole concentrations are used in the calculations. When reduced to the concentration of the neutral or unionized form of imidazole, using $pK = 7.10$, as described in the experimental part, it is seen that the drift in $K_{ER,I}$, with pH disappears, and $K_{ER,Icorr.}$ is perhaps independent of pH. The irregular variations are certainly within the limits of error. Indeed it is noticeable that the dissociation of DPNH and imidazole from the enzyme ($K_{E,R}$ and $K_{E,I}$), and the dissociation of I, R or O from ER and EI ($K_{ER,I}$; $K_{EI,R}$; and $K_{EI,O}$) are intensitive to pH changes. However, the dissociations of O from EO ($K_{E,O}$) and of I from EO ($K_{EO,I}$) are interdependent and pH sensitive, $K_{E,O}$ and

Table 16.

pH	$K_{E,I} \times K_{EI,R} = K_{E,R} \times K_{ER,I}$	$K_{E,I} \times K_{EI,O} = K_{E,O} \times K_{EO,I}$
6	0.95	212
7	1.10	233
8	1.49	292
9	1.95	292

Table 17. Experiments XII B and X B. Alcohol inhibition. Equilibrium experiments.

Q	$K_{ER,alc}$	$K_{E,alc}$ *	$K_{Ealc,R}$
7.7	40 mM	100 mM	0.12 μM

$K_{EO,I}$ changing in a reciprocal way due to the relation $K_{E,I} \times K_{EI,O} = K_{E,O} \times K_{EO,I}$ (eqn. 9) and the relative pH insensitivity of $K_{E,I}$ and $K_{EI,O}$. Table 16 shows the inverse relation between $K_{E,O}$ and $K_{EO,I\ corr}$. There may be an upward trend but the values are not strongly (if at all) pH dependent.

Several other nitrogenous compounds were preliminarily tested for their ability to form complexes with E or ER. Histidine seemed to form a weak complex ($K_{E,I} \approx 0.1$ M); piperazine was somewhat stronger. Pyridine was considerably stronger and formed both an EI and an ERI complex less strongly fluorescent than ER. Veronal, and particularly collidine, formed strong EI-complexes. Preliminary experiments with YADH and pyridine indicated complex formation similar to that with LADH. In kinetic experiments the *ionized* form of pyridine bases was stated to inhibit YADH [15]. Experiments with lactic dehydrogenase and imidazole gave no indication of any complex formation, which would support imidazole and certain nitrogen compounds being joined to the zinc of LADH or YADH, and lactic dehydrogenase not containing zinc in agreement with Pfleiderer [16].

Ethanol. It has been known for many years [4] that concentrations of ethanol higher than 10 mM cause considerable inhibition of the reaction with liver-ADH and DPN. This is the reason why in our kinetic experiments concentrations of ethanol higher than 6.1 mM were not exceeded*. It wast herefore considered interesting to investigate whether additions of relatively high concentrations of alcohol to ER caused any changes in fluorescence intensity and $K_{ER,app}$ indicating the formation of an ERI complex at pH 7.

Fig. 6 shows this to be the case. $K_{E,R,app}$ and Q both changing along what can be considered to be a monovalent dissociation curve with 50 % dissociation at around 40 mM. With increasing alcohol concentration, $K_{E,R,app}$ went down from 0.31 to 0.12 μM, and Q from 12.5 to 8, indicating that a ternary complex ERalc is formed.

The values of the dissociation constants are shown in Table 17. It was found that 1 M ethanol gave a 10 % fluorescence increase for DPNH and in calculating Q and D_{app} the effect of this on r and hence on the F values in the programmes had to be allowed for. In these experiments alcohol behaves like imidazole in forming complexes ERI, EI, and EIR (I = alcohol) and the dissociation constants were similarly calculated from the variation of Q ($K_{ER,alc}$) and D_{app} ($K_{E,alc}$ and $K_{Ealc,R}$) with alcohol concentration using eqns. 3—7. For eqn. 4, Q values taken from a smooth curve drawn through the experimental points were used to obtain Q_∞. $K_{ER,alc}$ is also the mid point of the dissociation curve (Fig. 6). E,alc * is denoted with an asterisk to distin-

* Winer nnd Schwert [16a] reported evidenec for the existence of a LADH-DPNH-alcohol complex being formed at acidic pH values.

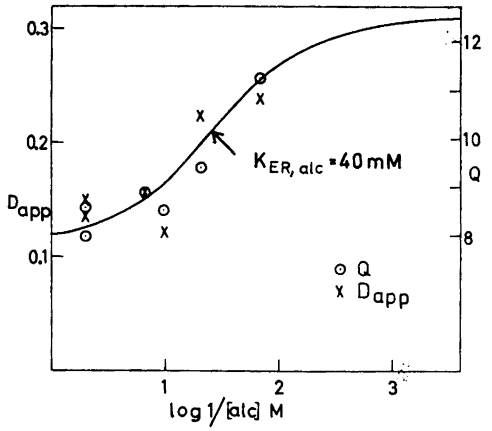

Fig. 6. Experiments with high [alcohol]. Variation of D_{app} and Q with alcohol concentration.

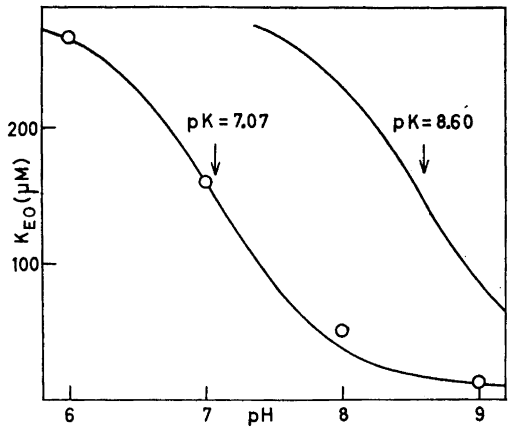

Fig. 7. Experimental values of $K_{E,O}$ at pH 6, 7 and 9 adapted to fit a dissociation curve with asymptotes 288 and 8.5 μM. pK 7.07. The titrable group in the free enzyme then has a pK of 8.60.

guish it from the ordinary, binary E,alc complex ($K_{E,alc}$ = 4.6 mM) described in Part III. The implications of these findings will be discussed together with some kinetic experiments in Part III.

DISCUSSION

$K_{E,R}$. The present values for K_{ER} on the whole agree with the previous ones obtained by both kinetic and equilibrium measurements [12]. The glycine effect described above should, however, be corrected for at pH 9 to 10. At pH 9 this reduced $K_{E,R}$ from 0.9 to 0.65 μM. Higher pH-values were not used in the present study, but it would be logical to assume that the glycine effect would be higher at pH 10 than at pH 9, since it probably is proportional to the concentration of $NH_2—CH_2—COO^-$, assuming that $—NH_2$, but not $—NH_3^+$ is coordinated with zinc.

In Table 15, $K_{E,R}$ is essentially pH independent. However, above pH 9, $K_{E,R}$ changes rapidly from 0.65 μM at pH 9 to near 5 μM at pH 10 where it is already half $K_{E,O}$ [12]. This sudden change with pH would seem to indicate that the pyridine ring in DPNH is bound to the protein by groups with a pK around 10. SH groups are known to add across the 2—3 position of DPNH model compounds but not DPN [19]. Previously the participation of SH groups in the binding of DPNH to LADH was suggested from the shift on addition of *p*-chloromercuribenzoate, of the 325 mμ peak of the binary complex to 340 mμ [17], although the mercurial could have liberated the DPNH by breaking down the enzyme's own tertiary structure rather than specific enzyme SH — coenzyme bonds. In the above, the protein SH groups would account for

the pH sensitivity of ER at alkaline reaction. The pH variation of $K_{E,O}$ is here considered due to a zinc-pyridinium rather than an SH-pyridinium interaction [4,15,20].

$K_{E,O}$. The new values for $K_{E,O}$ differ from the earlier ones partly because of the quenching effect of DPN on the fluorescence of ER. It should be noticed that the differences are largest in acid solution, decreasing towards pH 9, where there is complete agreement between the present value of $K_{E,O} = 16$ μM and the previous value 15 ± 1.4 μM, both in ~100 mM glycine buffer. The reason for this is obvious: at the low pH values $K_{E,R}$ is low, $K_{E,O}$ high, so that large concentrations of DPN of up to 1 000 μM or more were required to give suitable competition with DPNH. Therefore, the quenching through DPN caused large errors. At pH 9 the situation was the opposite; non-quenching DPN-concentrations around 10—20 μM DPN were high enough in these experiments.

The conclusion drawn in 1959 [12] that the postulated Theorell-Chance mechanism was half right and half wrong, with agreement between kinetic and equilibrium values at alkaline pH, disagreement at acid pH [12,18], must now in view of the higher $K_{E,O}$ values from equilibria at acid reaction be corrected to say that satisfactory agreement is observed at acid as well as alkaline pH, as discussed in Part I.

The $K_{E,O}$ values from 1959 conformed to a dissociation curve with a pH ~ 7.5 and already in 1951 [17] the redox potential-pH curve, calculated from the value of $K_{E,O}/K_{E,R}$, gave indication of a DPN-linked group in the enzyme with a p$K \approx 7.8$.

It was therefore of interest to analyse the present values somewhat closer. The general trend of the points indicated that towards extremely acid pH, $K_{E,O}$ would approach an upper asymptote, disregarding secondary effects that could enter into the picture at such high activity. Towards the alkaline side a lower asymptote must be assumed since $K_{E,O}$ cannot reasonably go down to zero, and the earlier value was 15 μM at pH 9 and 10 μM at pH 10, both in 100 mM glycine [12].

Since 3 values with a consistent trend can always be brought to fit a dissociation curve it was decided to use the values of $K_{E,O}$ at pH 6, 7 and 9, as probably being the most accurate ones for finding the upper and lower asymptotes and the pK value.

Since according to these assumptions the binary compound EO is not entirely dissociated at any of the pH values, DPN must be assumed to be attached to the enzyme by two or more bonds. In the case of DPN, but not DPNH, one of these is a titrable, acid group in the protein, contributing to the sta-

Table 18. Monovalent dissociation curve made to fit the experimental values of $K_{E,O}$ at pH 6, 7 and 9. $K_{E^-H^+,O} = 288$ μM, $K_{E^-,O} = 8.5$ μM, p$K = 8.60$.

pH	6	7	8	9
$K_{E,O}$ exp	266	160	51	12
$K_{E,O}$ calc	266	160	37	12

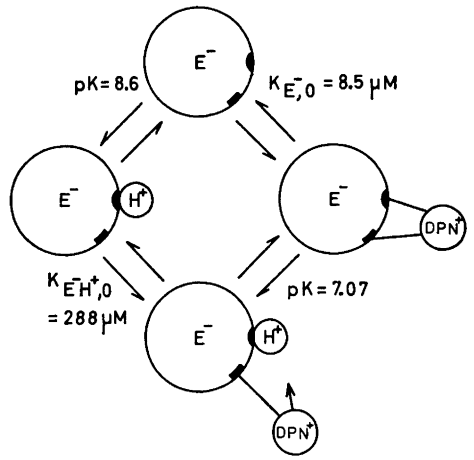

Fig. 8. The equilibrium between LADH, DPN and H^+ and the mode of attachment of DPN.

bility as a result of the coulombic attraction between a negatively charged group in the protein and the positively charged pyridine ring of DPN, as suggested previously. There would thus be a competition between protons and the pyridine ring for this negative group.

It was found that the $K_{E,O}$, values 266 μM (pH 6); 160 μM (pH 7) and 12 μM (pH 9) fitted with a dissociation curve if the acid asymptote was 288 μM, the alkaline one 8.5 μM and the pK of the acid group in the free enzyme = 8.60 and the pK of the same group in the binary EO complex was 7.07 (Table 18). The relation between these numbers is defined by the equation:

$$\log \frac{288}{8.5} + 7.07 = 8.60$$

The dissociation curves of the negative group in the free enzyme (pK 8.60) and in LADH—DPN (pK 7.07) are seen from Fig. 7. The value of $K_{E,O}$ for pH 8 falls close to the theoretical curve; $K_{E,O}$ exptl. = 51, $K_{E,O}$ required by theory = 37 μM. It must be noted that all the errors inherent in the values at 6, 7 and 9 are by this procedure imposed on $K_{E,O}$ at pH 8. Allowing small errors in all four constants could give a much closer approach to a dissociation curve within the limits of error for all four constants. The equilibrium and attachments assumed are illustrated in Fig. 8.

In Part IV it is concluded that Zn is bound by more than two bonds to the protein, where "more than two" is most likely 3. In the free enzyme the zinc seems to be bound in an octahedral complex[21]. A free zinc ion cannot readily attach more than 4 molecules of imidazole as unidentate ligands[2,3]. It is therefore logical to assume that a zinc atom with three bonds to the protein

cannot attach more than one molecule of imidazole, and indeed the equilibrium measurements could all be interpreted on the basis of assuming one molecule imidazole per binding site. The values for $K_{E,I}$ and $K_{ER,I}$ came out practically constant in the presence of from 3 mM to 100 mM imidazole (Tables 13 and 14).

The free bonds of the zinc in the free enzyme are very probably attached to water molecules in neutral or acid solution, and one or more would give hydroxo groups in alkaline solution. It seems entirely reasonable that this transition could occur with a pK of 8.60, as calculated. In the natural β-isomer of DPNH the pyridine ring is so close to the adenine that energy transfer takes place between the two [22,23], and since adenine is presumably bound to Zn, the pyridine ring must be rather close to the Zn as well. Coulombic attraction between the $-OH^-$ and the positively charged pyridine ring in DPN could explain the increase in stability of the DPN—ADH complex with increasing pH, which is not operative in DPNH—ADH. Introduction of imidazole instead of OH^- would eliminate this attraction effect, as is indeed observed.

In EO at high pH DPN uses all three bonds to the Zn, two for the adenine and one for $Zn-OH^--N^+ C_5H_5$. This last bond is the same that imidazole has to use for the formation of the ternary EOI complex. There is thus competition between imidazole and the positively charged pyridine ring for this bond. Alkaline reaction favours the hydroxo group formation, which stabilizes the bond to pyridine$^+$ but not to neutral imidazole. This explains why $K_{EO,I}$ increases with pH in the same proportion as $K_{E,O}$ decreases. The bonding of DPN to ADH in acid solution ($K_{E,O} = 288\,\mu M$) is about equally strong as DPN to ADH-imidazole ($K_{EI,O} = 440\,\mu M$): two bonds from adenine to Zn, the third bond holding water in the first case, neutral imidazole in the second. In addition to this other bonds between DPN and the protein may contribute to the stabilization of both.

In alkaline solution there is a parallel situation between ADH-DPN ($K_{E,O} = 8.5\,\mu M$) and ADH-DPN-caprate ($K_{EI,O} = 7\,\mu M$). The negatively charged caprate ion seems to have the same stabilizing effect as the hydroxo group at the zinc atom. Variations in the length of the carbon chain of fatty acids (C_1 to C_{15}) [1] have no great influence on $K_{EI,O}$, the charge effect being roughly the same in all of them. The dramatic changes in $K_{EO,I}$ with the chain length must reasonably be attributed to a lipofilic binding site.

For *iso*butyramide $K_{ER,I}$ and $K_{EI,R}$ when compared with $K_{E,I}$ and $K_{E,R}$ showed 66—70 fold mutual stabilization to operate between I and R. In the ternary complex EOI with DPN and caprate there is a 22—23 fold mutual stabilization. Imidazole which forms both ERI and EOI complexes on the contrary causes *de*stabilization. This is 6—7 fold for ERI, 3 fold for EOI at pH 7. With the DPNH complexes the destabilization is rather insensitive to pH changes, whereas with the DPN complexes the destabilization increased with pH due to the additional effect of the N^+-OH^--Zn interaction.

We think $K_{E,I}$ for imidazole is comparatively insensitive to pH changes, because imidazole with the free enzyme can attach to any of the free Zn bonds. The slight increase in $K_{E,I}$ with pH may result from the hydroxo group being more tightly bound to Zn than is a water molecule.

In the ternary complex ERI no charge effects operate between the neutral pyridine ring in DPNH and the neutral imidazole. As discussed in Part III we think that in ERI all three of the free Zn bonds are occupied, two by the adenine moiety and one by neutral imidazole, leaving no opportunity for pH effects to occur by formation of hydroxo groups in alkaline solution.

The increased fluorescence of ER compared with R has been attributed to the greater rigidity of R attached to the enzyme [20,28], and in particular to the interaction between the pyridine ring and zinc. Why there is a further increase in the ternary complexes with imidazole and with *iso*butyramide (but not with most of the other amides) we do not know yet.

Due to the stereospecific nature of the LADH reaction with regard to the plane of the pyridine ring [24,25] (it is also stereospecific towards the substrates) it has been suggested that the amide group in the pyridine ring gives the ring its stability by joining to the enzyme, possibly the zinc [4,26]. It is possible that the amide group is bound to the protein at a site other than zinc, but this is not necessary with LADH as the protein SH addition to pyridine in DPNH and the pyridinium-Zn bond in DPN would suffice to give the pyridine ring its stereospecific rigidity.

Further discussion on the bonding of coenzymes, substrates and inhibitors will be found in Part III.

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