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Dissociation Constants of the Binary Complex of Homogeneous Horse Liver Alcohol Dehydrogenase and Nicotiniumamide Adenine Dinucleotide

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The coupling of NAD^+ to homogeneous liver alcohol dehydrogenase (LADH) was studied. The resulting spectrophotometric change was followed in the ultraviolet region (main peak at 281 mμ) by means of double difference spectrophotometry. Extreme care was taken throughout to prevent alcohol contamination.

The absorption increase, induced by the coupling of β-nicotiniumamide dinucleotide ($\Delta\varepsilon$ at 281 mμ = 3.0 ± 0.1 $mM^{-1} \times cm^{-1}$) with LADH was not seen with the α-isomer or with β-nicotiniumamide mononucleotide or with β-nicotiniumamide dinucleotide phosphate. This absorption increase provided a means of a direct determination of the dissociation constants of the β-nicotiniumamide dinucleotide complex with homogeneous liver alcohol dehydrogenase over the pH range 6 to 10. The dissociation constants thus directly determined are in good agreement with those redetermined using the previous indirect spectrofluorimetric method.

Titration data indicate the presence of two independent and equivalent β-nicotiniumamide dinucleotide-binding sites per LADH molecule throughout the pH range 6 to 10. The dissociation constants determined at various pH values could be fitted to a monovalent dissociation curve with pK = 8.75 for the proton donating group of the free enzyme, confirming the previous assumption that the ternary complexes, enzyme-imidazole-β-nicotiniumamide dinucleotide and enzyme-fatty acid-β-nicotiniumamide dinucleotide, are analogous to the acid and alkaline extremes of the enzyme-β-nicotiniumamide dinucleotide binary complex.

The present paper deals primarily with the direct determination of the dissociation constants of the binary complex of homogeneous LADH and NAD^+ at different pH values.

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The shift of the 340 mμ absorption maximum of NADH * to 325 mμ upon coupling of NADH to LADH [2] was observed in 1951 and in the same year it was demonstrated that LADH had two coenzyme-binding sites per molecule.[3] These observations together with the subsequently observed fluorescence enhancement upon formation of the ER complex [4] made possible the determination of the dissociation and rate constants for the coupling of LADH and NADH.

Despite numerous trials, however, direct studies of the coupling of NAD^+ to LADH have been unsuccessful because in the comparatively high concentrations of both, necessitated by the large value of the dissociation constant of the LADH—NAD^+ complex (K_{EO}), some reduction of the NAD^+ occurred.

We have recently succeeded in removing residual reducing components, the primary source of these experimental difficulties, by employing electrophoretically homogeneous $LADH_E$ samples and by carefully controlled dialysis.[5] Using this LADH preparation of established homogeneity and the technique of double difference spectrophotometry [6] we have characterized the ultraviolet spectral changes associated with EO complex formation. These spectral changes, with a main peak at 281 mμ, have enabled us to make the first direct determination of the dissociation constants for the dissociation of NAD^+ from LADH at various pH values.

The present spectrophotometric approach is more straightforward than the previous equilibrium measurements in which the competitive relationship between NAD^+ and NADH [7,8] or between NAD^+ and *o*-phenanthroline (an established coenzyme-competitive inhibitor [9] for LADH) was used.

MATERIALS

Enzyme, $LADH_E$: Homogeneous $LADH_E$ was prepared according to a method that has been used in this laboratory for a number of years, referred to as "a modification of Dalziel's method".

The method of Dalziel [10] was followed up to the stage of crystallization which was performed from 8—10 % ethanol solution rather than the 6 % used by Dalziel. The material was then recrystallized from 6 % ethanol. This crystalline enzyme preparation, which, even after several recrystallizations, was never completely homogeneous on electrophoresis was further purified by chromatography on carboxymethyl cellulose (CMC).

About 2 g of enzyme was placed on a 3 × 50 cm column of CMC at + 3°C and developed with 500 ml of phosphate buffer, $\mu = 0.05$ and pH 6.0, at which pH practically all of the enzyme was retained on the column. A small amount of active material, apparently attached to some acid component, was usually eluted with this buffer. The development was then continued with phosphate buffer, pH 6.8, $\mu = 0.05$, until about 80 %

* The following abbreviations are used: LADH, liver alcohol dehydrogenase; NADH and NAD^+, reduced and oxidized nicotiniumamide dinucleotide, respectively; $NADP^+$, oxidized nicotiniumamide dinucleotide phosphate; NMN^+, oxidized nicotiniumamide mononucleotide; ADPR, adenosine diphosphate ribose. LADH, NAD^+, NADH, pyrazole, isobutyramide, fatty acid inhibitor, and imidazole are abbreviated as E, O, R, P, I, FA, and I′, respectively, in expressing dissociation constants and the form of binary and ternary complexes of LADH. To distinguish the steroid-inactive and homogeneous LADH used in the present studies from separate steroid-active alcohol dehydrogenase recently crystallized from horse liver,[1] the former LADH is expressed as $LADH_E$.

of the enzyme had emerged from the column. The rest of the enzyme was then eluted with phosphate buffer, pH 8.0, $\mu = 0.2$.*

In most preparations only a very small middle fraction of the pH 6.8 eluate was found to be homogeneous and a larger middle fraction, containing 50—60 % of the original activity, was therefore rechromatographed under the same conditions. The homogeneous LADH obtained from this chromatography amounted to 25—40 % of the enzyme initially charged on the first column. All other active but heterogeneous fractions from the chromatography were combined and subjected to rechromatography.

The homogeneous LADH was crystallized by initial dialysis against 10 % ethanol in phosphate buffer, pH 7.0 $\mu = 0.05$. The contents of the dialysis bag were then transferred to an Erlenmeyer flask and the ethanol concentration raised to 35 % by slow addition of chilled 90 % ethanol while continuously lowering the temperature to just above the freezing point. The crystals thus obtained are indefinitely stable at −18°.

Before use the enzyme crystals were collected by centrifugation, dissolved in a minimum volume of Na_2HPO_4, $\mu = 0.1$, with ammonia added to give pH 9.5 and thoroughly dialysed (for three days at 2° with 8 changes of the outer solution) as described in a previous paper.[5]

The concentration of the dialyzed enzyme preparation was determined spectrophotometrically by titrating $LADH_E$ with purified NAD^+ (see below) in the presence of excess pyrazole in pH 7.0, 0.1 μ,[11] phosphate buffer; the concentration of $LADH_E$ was expressed as N, the normality of the coenzyme-binding capacity per liter. The preparations used were found to be 100 % active on the basis of a specific extinction coefficient at 280 mμ of 0.455 (mg/ml × cm^{-1}) and a molecular weight of 84 000 per two coenzyme-binding sites.

The concentration of contaminating alcoholic components in the samples used in the present study, determined as ethanol by spectrofluorimetric assay,[5] was even lower than the value reported in the previous paper (0.1 mole of alcoholic components per coenzyme-binding site). This is probably due to improved techniques for protection against alcohol contamination.

Coenzymes and other chemicals: NAD^+, purchased from Sigma Chemical Co., (98 % coenzymatically reducible, on the basis of a millimolar absorbancy index of NAD^+ at 260 mμ of 18.0 ($mM^{-1} \times cm^{-1}$) [12] and of NADH at 338 mμ of 6.22 ($mM^{-1} \times cm^{-1}$),[13] was purified to essentially 100 % by DEAE-cellulose column chromatography according to Dalziel's procedure.[14] NADH was prepared enzymatically from the purified NAD^+ following Dalziel's procedure.[15] The NADH was found to be at least 98 % coenzymatically active and had an absorbancy ratio $A_{259}/A_{338} = 2.46$. The concentrations of NAD^+ and NADH were expressed in terms of the coenzymatically reactive portion as determined by the 338 mμ absorption.

All other coenzyme and coenzyme fragments were purchased from Sigma Chemical Co. β-NMN^+ and ADPR were used without further purification. Both α-NAD^+ and β-$NADP^+$ were purified according to Dalziel's chromatographic procedure.[14] The eluate of α-NAD^+ and β-$NADP^+$ used contained 1.0 % and an undetectable amount of β-NAD^+, respectively, as assayed by LADH. All chemicals were of analytical grade.

Water and buffers. As described in a previous paper,[5] glass-redistilled water was used to prepare all buffers and was carefully handled to avoid contamination by any alcohols, especially from laboratory air.

At pH 6.0 and 8.0, 0.1 μ phosphate buffers were used, whereas 0.1 M glycine-NaOH buffers were used at pH 9 and 10. A 0.1 μ phosphate-glycine NaOH buffer, pH 9.0, (containing 3.65 mM glycine as used in previous studies [5,8]) and 0.1 M Tris-HCl buffer, pH 10, were also used. The Tris, purchased from Sigma Chemical Co. and found to contain appreciable amounts of reducing substances, was repeatedly recrystallized from a warm saturated solution by cooling and evaporation.

* The final eluate, obtained with the pH 8.0 phosphate buffer, reported by Dalziel to contain electrophoretically homogeneous enzyme,[10] was found to be definitely inhomogeneous under our conditions. It is now apparent, as recently reported,[1] that the main component found in this last eluate is not normal LADH but a steroid active alcohol dehydrogenase of more basic character ($LADH_S$). Since $LADH_S$ is more soluble in ethanolic phosphate buffer than $LADH_E$ this could explain why the $LADH_S$ fraction was not detected in the electrophoretic analysis of Dalziel as his crystals were obtained with a lower concentration of ethanol.

All reagents prepared using glass-distilled water were found to be practically free from reactive alcoholic components by routine alcohol assay using yeast alcohol dehydrogenase (purchased from C. F. Boehringer und Soehne, Mannheim). The pH measurements were made at 23.5° using a Radiometer pH meter 25. The pH of the titrated mixtures of $LADH_E$ and coenzymes was measured with a microelectrode immediately after completion of the titration.

MEASUREMENT METHODS

All spectrophotometric measurements were carried out at 23.5°. The interaction of $LADH_E$ with NAD^+ was studied using the technique of double difference spectrophotometry previously described.[6,16] Spectra were recorded with a Beckman DK-2 ratio recording spectrophotometer or a Cary spectrophotometer, Model 14-A. The absorbancy changes were measured a few minutes after the gentle addition, with a plastic stirring bar, of NAD^+ to an LADH solution. A stable spectrophotometric reading was readily attained.

In any spectrophotometric experiments on the EO complex, a correction must be made for reduction of NAD^+. Though this can be largely minimized, it is still observable, even with carefully dialysed electrophoretically homogeneous samples. This correction was carried out spectrofluorimetrically in the following simplified manner. The enzyme solution in one of the reference cuvettes used in the double difference spectrophotometric measurements, to which had been added a volume of buffer equal to the volume of NAD^+ solution added to the sample cuvette, was transferred to a non-fluorescent cuvette. An amount of NAD^+ equal to the total amount that had been added in the titration experiment was then added to this solution, and the increase in fluorescence emission intensity at 410 mμ (excited at 330 mμ) was determined spectrofluorimetrically.[17] Assuming that the increase in the fluorescence intensity is due solely to the bound form of R, which is equal to the reacted portion of alcohol, the amount of bound NADH determined spectrophotometrically ($= \tau$ (μM)) was taken as a direct measure of the reacted portion of alcohol at pH 6—9. This assumption is justified by the small values of K_{ER} (0.23—0.65 μM [8]) between pH 6—9 and the relatively high concentration of enzyme used in the present study.* Using the difference millimolar extinction coefficient, at 281 mμ, of the EO and ER complex as given in the text, we have

$$EO\ (\mu N) = \frac{10^3}{3}\ (\Delta A_{281} - \tau \times 0.00138)$$

where $E_f = E_t - EO - \tau$ and $O_f = O_t - EO - \tau$ (the suffixes f and t denote free and total enzyme or NAD^+, respectively).

In Tables 2—5 and 7 the final dissociation constants were calculated from these corrected values of EO for pH 6—9. A different method of correction, however, was applied to the data obtained at pH 10, where the K_{ER} value is greater and is comparable to K_{EO} (both nearly equal to 10 μM as

* Binding of most of the NADH under these conditions can be seen even at pH 9.0, as shown in the data presented in Table 1 of Ref. 5.

Table 1. Summary of K_{EO} values at pH 6–10 as determined by direct spectrophotometric titration, and by competition with NADH, fluorimetrically.

pH	6.0	7.0	8.0	9.0	10
Spectrophotometrically determined K_{EO}, μM	325,** 350,* 377 **	(107),* 123, 130 ** 137, 141 ***	24.5,** 30.1,* 35.7 **	9.0, 9.6 ** (14.6, 17.1) *	5.3,*** 5.5,** 5.8 (6.6, 7.6) *
K_{EO}, μM average	351	133	30.1	9.3	5.5
Fluorimetrically determined K_{ER}, μM	0.07 ***	0.20 **	0.28 ***	0.46 ***	6.6 **
Q_{ER}	10.8	12.7	12.1	11.4	16.0
K_{ER} app., μM	0.18	0.37	0.85	2.56	22.95
Q_{ER}	10.5	11.0	11.6	11.7	15.7
with [O], μM	500	125	70	50	14.4
Calculated K_{EO}, μM	322 ***	132 **	34.3 ***	11.0 ***	5.9 **

The pH values measured after each titration showed a fluctuation of ± 0.05 pH units from each value given in the table. The two values in parenthesis at pH 9 and 10 were determined in 0.1 M glycine NaOH buffer whereas the other two values at pH 9.0 were determined in phosphate (0.1 μ)-NaOH-glycine (3.64 mM) buffer and the three values at pH 10 were determined in Tris-HCl (0.1 M) buffer. Asterisks denote the $LADH_E$ preparation used (*: E-4,**: F-3 and ***: F-5).

previously determined by spectrophotometric experiments [18]). Retaining the assumption that the NADH is produced immediately after the first addition of NAD^+ to the LADH solution, the binding of R by E at this pH can now be considered as being under the competitive influence of the O present. Therefore, $E + R \rightleftharpoons ER$ and $E + O \rightleftharpoons EO$,

$$K_{ER} = \frac{E_f \times R_f}{ER} = \frac{E_f \times R_f}{EO} = K_{EO} * \text{ where}$$

$$E_f = E_t - ER - EO$$

$$O_f = O_t - EO - \tau$$

These equations, together with the relationship

$$ER\ (\mu N) \times 1.38 + EO\ (\mu N) \times 3.0 = A_{281} \times 10^3$$

$$\text{give } EO = \frac{A \times 10^3 \times (O_t - \tau)}{3\ O_t - 1.62}$$

$$\text{and } ER = \frac{\tau \times \Delta A \times 10^3}{3\ O_t - 1.62}$$

Data obtained at pH 10, corrected using the above equations, are listed in Table 6.

Table 2. Titration of $LADH_E$ (E-4) with NAD^+ at pH 6.0 in 0.1 μ phosphate buffer.

Addition of O No.	O_t (μM)	EO	E_f	O_f	K_{EO}
1	75	24.7	183.1	41.6	341
2	150	45.3	162.5	100.5	360
3	225	63.4	144.4	157.4	358
4	300	80.0	127.8	215.8	340
				Average	350 μM

Conditions: 212 μN $LADH_E$ plus 4 × 75 μM NAD^+ in a cuvette of 5 mm optical path; pH 6.02 at 23.5°C.
$\Delta\varepsilon_{281} = 3.0$ ($mM^{-1} \times cm^{-1}$) was assumed at this pH.

Spectrophotometric changes upon formation of the EO complex. The light absorption changes caused by the coupling of NAD^+ to LADH at pH 10 are recorded in Fig. 1 (A): these are compared to those induced by the coupling between LADH and NADH (B) and the coupling between LADH and ADPR (C), both recorded at pH 7.0.

* The validity of the assumption that $K_{ER} = K_{EO}$ at pH 10 can be seen not only from the previous data [18] but from the newly determined values (6.6 and 5.5 μM, respectively) as recorded in Table 1.

The instability of the ER complex, as shown by the gradual decrease in its fluorescence intensity, has been previously attributed to a degradation reaction. This instability has recently been investigated in detail, and a report will be published elsewhere.[19] Accurate spectrophotometric experiments on ER formation are difficult because of this instability. Thus the values $\Delta\varepsilon$ ($mM^{-1} \times cm^{-1}$) at 281, 302, and 355 mμ, which are needed for correction in the present spectrophotometric experiments on EO complex formation, were determined by extrapolating the time course of the spectral change to time 0. The extinction coefficients thus determined were 1.38, 1.36, and 2.5 ($mM^{-1} \times cm^{-1}$) at 281, 302, and 355 mμ, respectively. These values are very close to those obtained previously [16] from the difference spectrum observed when R was added to LADH in the presence of excess isobutyramide(I) to form the highly fluorescent complex ERI. $\Delta\varepsilon$ ($mM^{-1} \times cm^{-1}$) at 281 mμ was constant over the range pH 6—10. The identity between these two difference spectra has already been noted.[16] Our studies on the modification reaction of NADH have shown that excess I stabilizes R in the ERI complex so that the spectrum of the ERI complex was found to be indistinguishable from that of the binary complex ER, extrapolated to mixing time zero.

The spectrophotometric change induced in our homogeneous LADH preparation by ADPR at pH 7.0 was found to be identical with that recorded in an earlier paper ($\Delta\varepsilon$ at 281 mμ = 1.3 ($mN^{-1} \times cm^{-1}$)).[16]

The spectrophotometric change due to EO complex formation was found by correcting for minute amounts of contaminating alcohol, which reacts with NAD^+ to give NADH. The amount of contaminating alcohol was determined by spectrofluorimetric assay using the simplified method described above. The presence of contaminating alcohol alters the spectrum particularly above 310 mμ, since the ER formed has a relatively high absorption ($\varepsilon_{325} = 5.8$ ($mN^{-1} \times cm^{-1}$))[2] in this region.

A more direct method for determining the maximum decrease of the absorbancy at 325 mμ, the decomposition of the ER complex induced by the addition of 1 mM acetaldehyde, was tested. Addition of acetaldehyde caused an immediate small decrease in the absorbancy around 325 mμ due to the oxidation of ER. This was immediately followed by an increase in the absorbancy possibly due to acetaldehyde dehydrogenase activity of $LADH_E$.[20] Thus, there remains some uncertainty in the spectrum of EO formation above 310 mμ, and this region is plotted as a broken line in Fig. 1 (A).

The spectral change upon EO complex formation was greatest at 281 mμ with $\Delta\varepsilon = 3.0 \pm 0.1$ ($mN^{-1} \times cm^{-1}$). This difference extinction coefficient at 281 mμ was repeatedly determined, at pH 7, 8, 9, and 10, by adding increasingly larger excesses of NAD^+, in a cuvette of 5 mm or 1 mm optical path, to achieve apparent saturation of the absorbancy increase. Saturation was not attainable at pH 6.0, since the complex at this pH is highly dissociated. Thus the value of the difference extinction coefficient at 281 mμ, determined at pH 7 to 10, was assumed to be applicable also at pH 6.0. This value should be compared to 1.38 ($mM^{-1} \times cm^{-1}$) for ER formation and 1.30 ($mM^{-1} \times cm^{-1}$) for EA formation, both determined at 281 mμ. The absorbancies of the subpeaks at 295 and 301 mμ appear to be pH-dependent, and the ratio A_{301}/A_{281}

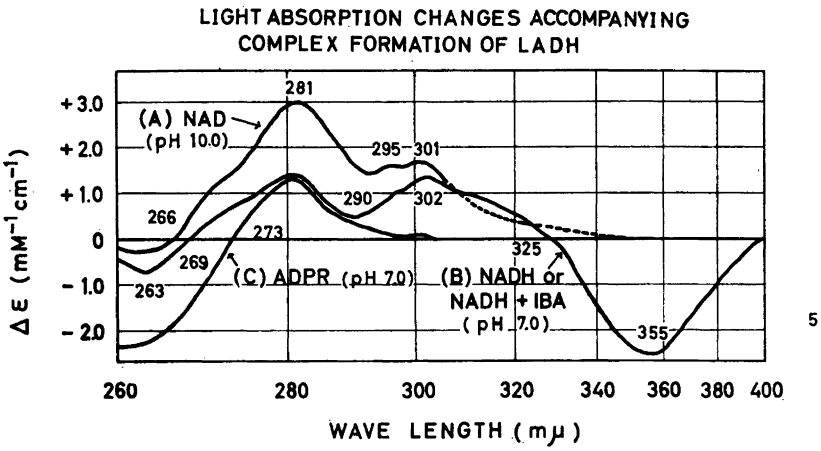

Fig. 1. Light absorption changes accompanying binary complex formation between LADH and (A) NAD^+, (B) NADH, and (C) ADPR.

Double difference spectra were recorded at 23.5°C in 0.1 M glycine-NaOH buffer, pH 10.0, for (A) and in 0.1 μ phosphate buffer, pH 7.0, for (B) and (C), at the following concentrations of reactants: (A) 100 μN $LADH_E$ plus 175 μM NAD (92.5 μN EO was assumed to be present as calculated from the value of K_{EO} determined in the present buffer (Table 1): (B) 80 μN $LADH_E$ plus 80 μM NADH (an identical and stable double difference spectrum was also obtained in the presence of 0.1 M isobutyramide): (C) 50 μN $LADH_E$ plus 130 μM ADPR. (42.4 μN of EA complex was assumed to be formed on the basis of a K_{EA} value of 16 μM [9]). The ordinate scale is based upon the calculated values of difference extinction coefficients of these LADH binary complexes.

decreases with decreasing pH; the main peak at 281 mμ associated with EO complex formation is pH independent. The difference extinction coefficients at 301 mμ, determined as the average from 3 titrations at each pH, were 0.86, 0.96, 1.09, 1.18, and 1.66 ($mM^{-1} \times cm^{-1}$) at pH 6, 7, 8 9, and 10, respectively.

Analogous to the identity of the difference spectrum of ER and EIR at pH 7.0, the difference spectrum observed for EFAO (FA = caprate, $K_{EFA,O} = 7$ μM, pH independent) was virtually indistinguishable from that of EO. The spectral change associated with the coupling of NAD^+ to LADH was expressed as the $\Delta\varepsilon$ per bound NAD^+. The amount of bound NAD^+ was determined by correction for free NAD^+ and E. As recorded in Fig. 2, the amount of bound NAD^+ is compared both with free and bound NAD^+ in the presence of excess pyrazole, all recorded at pH 10. The 301 mμ peak is greatly enhanced by the tight coupling of NAD^+ to $LADH_E$ in the presence of pyrazole. No spectral changes were observed when NAD^+ was replaced by even higher concentrations of catalytically inactive coenzyme moieties or analogues such as β-NMN^+, α-NAD^+ and β-$NADP^+$. A mixture of β-NMN^+ and ADPR produced absorption changes identical with those induced by ADPR alone. In addition, no detectable difference in absorption was observed in titrations of LADH with β-NAD^+, at pH 10, whether or not 100 μM α-NAD^+

Table 3. Titration of $LADH_E$ (F-3) with NAD^+ at pH 7.0 in 0.1 μ phosphate buffer.

Addition of O No.	O_t (μM)	EO	E_f	O_f	K_{EO}
1	60	30.2	159.8	23.8	126
2	120	58.7	131.3	55.3	123
3	180	78.0	112.0	96.0	137
4	240	100.0	90.0	134.0	121
5	300	107.0	83.0	187.0	145
				Average	130 μM

Conditions: 196 μN $LADH_E$ plus 5 × 60 μM NAD^+ in a cuvette of 5 mm optical path; pH 6.97 at 23.5°C.

was added; this suggests that LADH is stereospecific for β-NAD^+, in agreement with the absence of fluorescence enhancement upon addition of α-NADH to LADH [21] and the kinetic inactivity of α-NADH.[22]

Table 4. Titration of $LADH_E$ (E-4) with NAD^+ at pH 8.0 in 0.1 μ phosphate buffer.

Addition of O No.	O_t (μM)	EO	E_f	O_f	K_{EO}
1	15	7.67	47.7	5.2	32.4
2	30	16.00	39.4	11.9	29.3
3	45	22.67	32.7	20.2	29.1
4	60	28.00	27.4	29.9	29.1
5	75	31.70	23.7	41.2	30.8
				Average	30.2 μM

Conditions: 57.5 μN $LADH_E$ plus 5 × 15 μM NAD^+; pH 8.02 at 23.5°C.

Determination of the dissociation constants for NAD^+ coupled with LADH as a function of pH. Table 1 is a summary of K_{EO} values determined at pH 6—10. As summarized, one set of K_{EO} values was determined at pH 6—10 with the same enzyme preparation (E-4). Additional titration experiments, carried out with different enzyme preparations, indicated that the titration with preparation E-4 gave values close to average values at pH 6 (Table 2) and pH 8 (Table 4). Since the value obtained with E-4 at pH 7.0 (K_{EO} = 107 μM) was lower than the four values determined with different preparations, titration data obtained with preparation F-3 is given in Table 3.

Theorell and McKinley-McKee [8] found that the glycine used for buffering at alkaline pH formed a binary complex with LADH. The K_{EO} values deter-

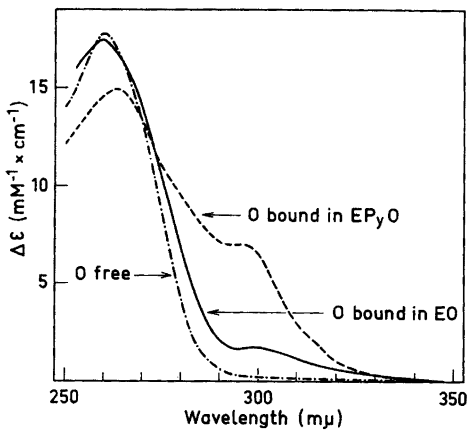

Fig. 2. Absorption spectra of the free and bound forms of NAD^+.

Measurements were carried out with 150 μN $LADH_E$, 30 μM NAD^+, and 1 mM pyrazole in 0.1 M glycine-NaOH buffer, pH 10. $O_{(bound)}$ is the difference spectrum of EO minus E (bound O assumed to be 28.3 μM on the basis of the value of K_{EO} in the present buffer (Table 1) and corrected for free NAD^+): $O_{bound\ in\ EPO}$ is the difference spectrum of EPO minus P (all O assumed to be in the bound form due to the small value of $K_{EP,O}$ = 0.1 μM).

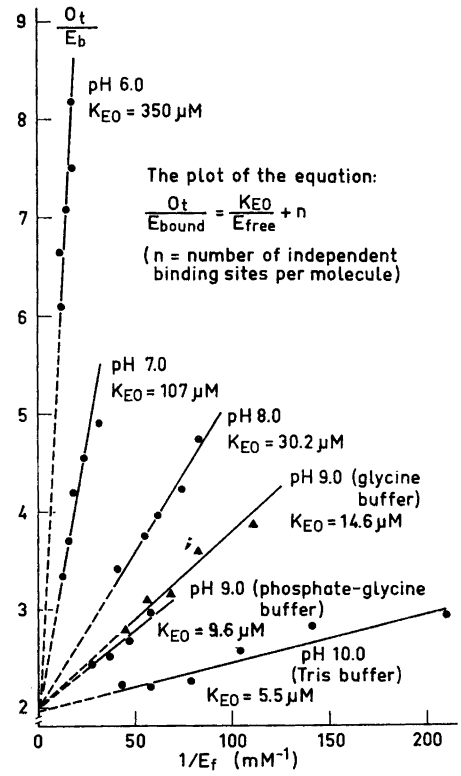

Fig. 3. Plot of the equation $O_t/E_b = (K_{EO}/E_f) + n$ at pH 6—10. (n = number of independent binding sites per molecule: enzyme concentration expressed as molarity). All plots are least mean square lines determined from the titration values, some of which are given in Tables 2, 4, 5, and 6.

mined by them with indirect spectrofluorimetric titration increased with increasing glycine concentration, especially above 81 mM. Thus the K_{EO} values at pH 9 and 10, determined in 0.1 M glycine buffer with E-4, were redetermined with preparation F-3 using a phosphate-glycine-NaOH buffer at pH 9.0 (containing only 3.64 mM glycine as used by Theorell and McKinley-McKee [8]) and with 0.1 M Tris buffer at pH 10. In titrations listed in Tables

Table 5. Titration of $LADH_E$ (F-3) with NAD^+ at pH 9.0 in 0.1 μ phosphate-NaOH-glycine (3.64 mM) buffer.

Addition of O No.	O_t (μM)	EO	E_f	O_f	K_{EO}
1	26.2	18.0	105.0	1.2	7.2
2	52.4	41.3	81.7	4.1	7.9
3	78.6	63.3	59.7	8.3	7.8
4	104.8	78.0	45.0	19.8	11.4
5	131.0	88.3	34.7	35.7	13.9
				Average	9.6 μM

Conditions: 130 μN $LADH_E$ plus 5 × 26.2 μM NAD^+; pH 8.97 at 23.5°C.

5 and 6, somewhat lower values of K_{EO} were obtained in the presence of a lowered concentration of glycine (3.6 mM instead of 0.1 M at pH 9.0) and in Tris buffer at pH 10. Another titration at pH 9 in phosphate 3.6 mM glycine-NaOH buffer, but with a different enzyme preparation, gave a K_{EO} value of 9.0 μM. At pH 10, the average value of K_{EO} obtained from three titrations in 0.1 M Tris buffer was 5.5 μM.

As a check on the newly determined dissociation constants of EO, both K_{ER} and K_{EO} (indirectly determined from the K_{ER} values obtained in the presence of NAD^+) were determined by spectrofluorimetric titration at pH 6—10 with preparations F-3 and F-5. The values obtained are listed in Table 1. The K_{ER} values obtained in this pH range appear to be smaller than the values previously determined [7,8] by the same method. K_{EO} values obtained by the indirect fluorimetric method are in good agreement with those directly determined by double difference spectrophotometry.

Coenzyme-binding state. Since first investigated in 1951 [2] it has been an open question of particular importance whether 1 or 2 moles of NADH couple

Table 6. Titration of $LADH_E$ (F-3) with NAD^+ at pH 10 in 0.1 M Tris-HCl buffer.

Addition of O No.	O_t (μM)	EO	ER	E_f	O_f	K_{EO}
1	12.5	9.1	2.05	46.35	1.1	5.6
2	25.0	20.0	2.02	35.48	2.7	4.7
3	37.5	29.9	1.95	25.65	5.3	4.6
4	50.0	36.3	1.75	19.45	11.4	6.1
5	62.5	41.6	1.59	14.31	18.6	6.4
6	75.0	46.5	1.47	9.53	26.2	5.4
					Average	5.5 μM

Conditions: 57.5 μN LADH plus 6 × 12.5 μM NAD^+; pH 10.2 at 23.5°C.

with one mole of LADH at pH 10.0. This coupling has been investigated utilizing a ternary ligand such as isobutyramide [23] and pyrazole.[11] In these complexes, the ratio LADH:coenzyme:ternary ligand = 1:2:2 has been well established. Hence, it is essential, in the case of a weakly coupled complex such as EO, to show whether one NAD^+ is bound comparatively tightly, or two NAD^+ are bound somewhat loosely. This can be determined from Fig. 3. Data obtained with preparation E-4 (for pH 6—9) and F-3 (for pH 10) and summarized in Table 1 are plotted according to the general equation derived originally by Klotz [24] (see below). This equation has been used to determine the binding of coenzyme to yeast [25] and swine muscle triose phosphate dehydrogenase.[26]

If there are n independent binding sites, the original equation [24] can be rearranged to

$$\frac{O_t}{E_b} = \frac{K_{EO}}{E_f} + n$$

E_b = fraction of enzymatic sites binding NAD^+ multiplied by the total enzyme concentration, expressed in molarity, and E_f = total enzyme concentration minus E_b, expressed in molarity.* A plot of O_t/E_b as ordinate against $1/E_f$ as abscissa is linear if the binding sites are equivalent and independent. The ordinate intercept is n (number of binding sites per molecule) and the slope is K_{EO}. As can be seen from Fig. 3, straight lines, determined by the method of least squares yield intercepts very close to 2 in all cases. This clearly indicates that two equivalent and independent binding sites are functioning throughout the range pH 6—10. The K_{EO} values determined from the slopes were identical to those presented in Tables 1, 2, 4 and 6.

DISCUSSION

Ultraviolet spectral changes induced with NAD^+-coupling. Changes in ultraviolet absorption associated with complex formation between LADH and ADPR, LADH and NAD^+ plus caprate, and LADH and NADH plus isobutyramide, were first studied by Theorell and Yonetani,[16] and the dissociation constant of the coenzyme or the coenzyme analogues of these complexes was determined. The spectral changes in all cases involve an absorption increase with a maximum at 281 mμ and a decrease in the region of 260 mμ. Studies of LADH plus NAD^+ mixtures have now been made possible by the use of thoroughly dialysed, electrophoretically homogeneous LADH samples.

A rigorous explanation of the ultraviolet absorption changes associated with the complex formations described in this paper will require further investigation. However, absorption changes induced by complex formation have recently been reported for other NAD^+-linked enzymes such as swine muscle triosephosphate dehydrogenase.[26] It is of particular interest to see

* It should be noted that E_b and E_f in this equation denote molarity of enzyme, whereas the symbol E otherwise denotes normality, *i. e.* 2 × molarity, of enzyme.

to what extent the spectral changes reported here are common to other NAD$^+$-linked dehydrogenases.

Any explanation for the spectral changes which occur upon complex formation must consider not only the involvement of multiple ultraviolet-absorbing groups in both LADH and NAD$^+$ molecules but also conformational changes that occur in the protein. The appearance of such a complex spectrum is essentially a reflection of conformational changes resulting from the interaction between LADH and NAD$^+$. Our data on the amino acid composition of LADH show that the LADH molecule contains 8 tyrosine, 4 tryptophan and 38 phenylalanine residues,[1] all of which could account for the changes in the ultraviolet spectrum reported here. In fact, light absorption changes in the ultraviolet region, of the magnitude found in our present experiments have been shown to follow conformational changes in the free $LADH_E$ protein. These conformational changes can be induced by changes in the tyrosine ionization due to pH changes, by addition of a denaturant such as urea, or by release of zinc from the enzyme protein, with simultaneous inactivation, upon acidification.[27] Conformational changes upon formation of the ternary complex of $LADH_E$ with coenzyme and inhibitors have already been suggested [23] and have been demonstrated by optical rotatory dispersion [28] and X-ray crystallographic methods.[29] *

For a quantitative comparison of the spectra recorded in Fig. 1, all of the peaks at 281 mμ, together with the negative peak around 260 mμ, appear to be primarily due to the interaction of LADH with the ADPR moiety.** Subpeaks between 295—302 mμ presumably result from the coupling of the nicotiniumamide moiety with $LADH_E$, as has already been suggested [16] since these peaks do not accompany the spectral change seen upon binding ADPR.

The 2.3-fold enhancement of the absorption change at 281 mμ upon EO formation over that obtained with EA formation, needs to be considered further. A possible explanation is given by assuming interaction between the "activity" group (zinc site for nicotiniumamide moiety) and the "specificity" group (ADPR site) of LADH, mediated by a NAD$^+$ molecule joining these two binding sites of the LADH molecule. This seems reasonable if the folded structure of NAD$^+$ bound over the coenzyme-binding sites is considered, where the adenine and nicotiniumamide moieties are arranged to be in juxtaposition. This suggests that both coenzyme-binding sites in LADH are proximally located to allow this interaction. α-NAD$^+$, which is incapable of assum-

* We have recently found (Zeppezauer *et. al.*, *Acta Chem. Scand.* **21** (1967) 1099) that the formation of ER from E + R is accompanied by the same change from orthorhombic to monoclinic symmetry as the formation of the ternary complexes ER-isobutyramide and EO-pyrazole.

** Prof. B. R. Rabin, University College, London, in a letter of Sept. 27, 1966 kindly informed one of us (H. Theorell): "I have been looking again at the difference spectrum which is produced on interaction of LADH and ADP-ribose reported by yourself and Yonetani. Dick Fisher in our laboratory has just measured the difference spectrum produced by taking AMP from pH 4.6 to an acid environment. You will be interested to hear that his difference spectrum is almost identical to the one you report. This strongly suggests that the adenine binding site of LADH is similar to that of RNase and that the interaction involves protonation of N-1 of the purine by an acid group in the protein. I would think a lysine residue is a very good candidate."

ing a folded hairpin structure, does not induce any absorption increase at 281 mμ with LADH.

The appearance of the well-known broad peak around 360 mμ when NAD^+ is coupled with muscle triosephosphate dehydrogenase has been attributed to a charge transfer between the pyridinium ring of NAD^+ and amino acid residues of the enzyme protein.[30] The 360 mμ peak, however, is not seen with our $LADH_E$ preparation.

The interesting hypothesis has been advanced that the absorption change, with a main peak at 272 mμ, of the complex of muscle triosephosphate dehydrogenase and NAD^+ is due to a mutual perturbation between the nicotinium and adenine rings of a common NAD^+ molecule, when the NAD^+ molecule is bound to the dehydrogenase.[31] This hypothesis, however, seems unlikely in our case because a similar absorption change around the main peak at 281 mμ was shown to be induced by ADPR alone.

The assignment of the subpeaks between 295—302 mμ is uncertain. Similar spectral changes in this region of a few times larger magnitude have been observed upon formation of the EPO type ternary complex. These spectral changes are presumably caused by the interaction of Zn^{2+} in $LADH_E$ with the nicotinium moiety of NAD^+ mediated through a third ligand such as pyrazole. The subpeaks between 295—302 mμ are not due to the interaction between the ADPR moiety of NAD^+ and LADH. They are enhanced as the binding of NAD^+ is fortified, either by an increase in pH or, particularly, by the presence of pyrazole (Fig. 2). All of these facts suggest that the subpeaks reflect an interaction between the nicotinium moiety of NAD^+ and the enzyme Zn^{2+}, irrespective of the kind of ligands (OH^-, COO^- or pyrazole) interposed. Judging from the (apparent) absence of interaction between LADH and NMN^+, alone or in the presence of ADPR, the pyridinium moiety must be bound to the ADPR moiety to achieve the proper steric arrangement between the pyridinium moiety and the enzyme Zn^{2+}. As described below, this interaction between pyridinium and the enzyme Zn^{2+} mediated by an interposed OH^-, is shown to be the main chemical event causing the pH dependence of the K_{EO} values.

Further spectrophotometric studies correlating conformational changes in the LADH molecule occurring on coenzyme-binding with simultaneous spectral changes will be of interest.

Dissociation constant of EO complex determined. As Table 1 shows, the newly determined values of K_{EO} deviate somewhat from the previous values determined by various indirect equilibrium measurements. However, evaluation of the new values should be made considering the directness of the method, *i.e.* the change is induced by the addition of NAD^+, and the LADH samples employed throughout were completely homogeneous. Also, the purity of NAD^+ has been improved; NAD^+ of approximately 90 % purity was used several years ago and may have contained coenzyme-competitive inhibitors such as ADPR.

The number of titration experiments at each pH is insufficient to allow a statistical treatment of the data. However, as listed in Table 7 where all K_{EO} values determined by various methods are summarized, an increase in the difference between the largest value of K_{EO} at pH 6.0 and the smallest value of K_{EO} at pH 10.0 is evident.

Table 7. Summary of experimentally determined K_{EO} values of $LADH_E$.

pH 6	7	8	9	10	Method employed	Authors
226	123	71	28 *	18 *	Kinetics ($= k_2'/k_1'$)	Theorell *et al.* (1955) [17]
107	84	28	15 **	10 **	Fluorimetric titration of E with R in the presence of NAD^+	Theorell and Winer (1959) [7]
—	141	—	16 **	—	Kinetics ($= k_2'/k_1'$)	Theorell and McKinley-McKee (1961) [8]
266	160	51	12 **	(8.5) ****	Fluorimetric titration of E with R in the presence of NAD^+	Theorell and McKinley-McKee (1961) [8]
313	137	43	9.1	3.9	Kinetics (Φ_1'/Φ_0)	Dalziel (1963) [32]
—	150	—	—	16 *	Spectrophotometric titration of E with *o*-phenanthroline in the presence of NAD^+	Yonetani (1963) [9]
351	133	30.1	9.3 **	5.5 ***	Direct spectrophotometric titration of homogeneous $LADH_E$ with NAD^+	Present data (1967)

Phosphate buffers were used, except at pH 9 and 10 where a glycine-NaOH buffer (*), phosphate-NaOH-glycine buffer (**) and Tris-HCl buffer (***) were used. The value (****) was calculated by assuming that the values already determined at pH 6–9 fit a monovalent dissociation curve with a $pK = 7.07$.

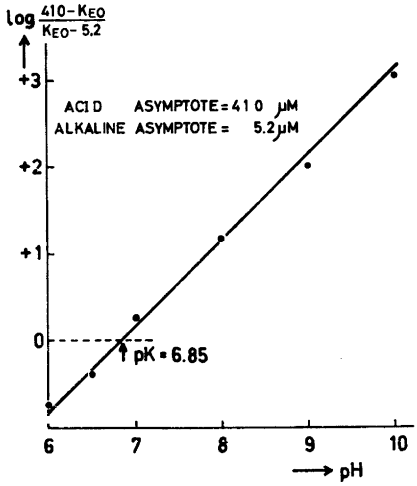

Fig. 4. Experimental values of K_{EO} at pH 6—10 which have been fit to a monovalent dissociation curve with asymptotes assumed at 410 μM and 5.2 μM. The average values determined by the present direct method at pH 6—10, as summarized in Table 1, together with a single value of K_{EO} = 296 μM obtained at pH 6.5 with preparation F-3, have been equally weighed in determining the best set of acid and alkaline asymptotes. The plotted line was determined from a least mean square fit to these six points. As indicated in the figure, the pK of the EO complex was determined to be 6.85.

In Fig. 4 the newly determined average values of K_{EO}, at various pH values, are plotted logarithmically assuming a monovalent dissociation curve with acidic and alkaline asymptotes of 410 μM and 5.2 μM, respectively, instead of the values used by Theorell and McKinley-McKee (288 and 8.5 μM, respectively) in a similar treatment of their data.[8] The plot obtained is compatible with a monovalent dissociation ($n = 1 \pm 0.013$). The pK of the EO complex, determined from this plot, is 6.85, and this gives, for the pK of the proton-donating group of the free LADH, 8.75, as determined from the equation:

$$\log \frac{410}{5.2} + 6.85 = 8.75$$

These values should be compared with the values proposed by Theorell and McKinley-McKee of 7.07 and 8.60.[8] The present values of K_{EO} at varied pH are in good agreement with the previous proposal that the pH variation of K_{EO} is due to a zinc-pyridinium rather than to a SH-pyridinium interaction. Dalziel has suggested [32] that there is an involvement of more than one group with pK's of approximately 8 and less than 6. These values were estimated from the linear increase of K_{EO}, as determined kinetically, from pH 8 (43 μM) to 6 (313 μM). This possibility now seems unlikely in view of the compatibility of our six experimental points assuming a single monovalent dissociation curve with a greater span between acid and alkaline asymptotes (pH 6 to 10).

Fig. 5 depicts a schematic representation of the equilibrium between LADH, NAD^+, and H^+ ion and a possible mode of attachment of NAD^+. Plotting the data according to the equation of Klotz [24] demonstrates the presence of two independent and equivalent NAD^+-binding sites per molecule throughout the pH range 6 to 10. In Fig. 3, the straight line drawn from the points at pH 6 towards a y-intercept of 2 suggests that the value $\Delta\varepsilon = 3.0$ ($mM^{-1} \times cm^{-1}$) assumed for the EO complex at pH 6 is correct.

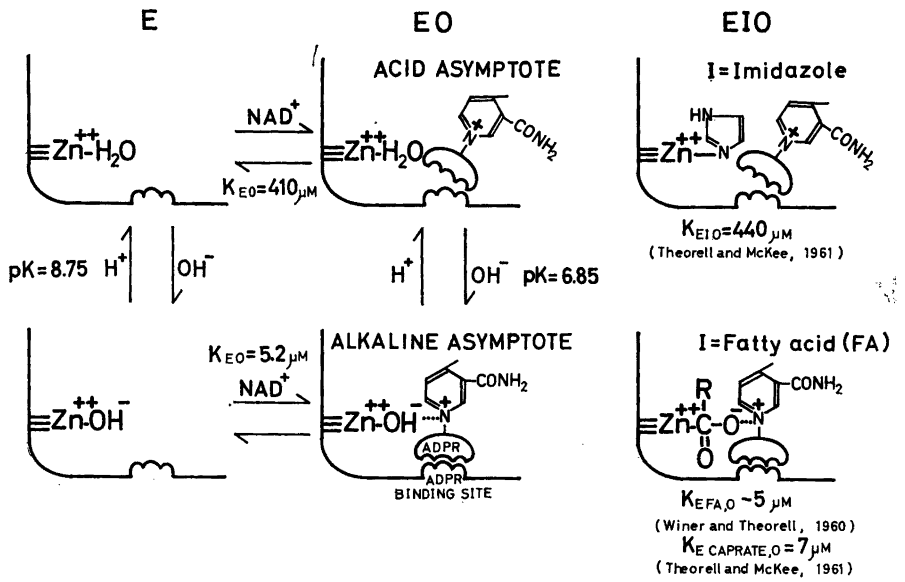

Fig. 5. The equilibrium between LADH, NAD^+, and H^+: the mode of attachment of NAD^+.
In addition to a Zn^{2+} and ADPR-binding site, the LADH molecule has a lipophilic binding site which is disregarded in the present schematic representation. The other bonds of Zn^{2+}, thought to be occupied by a water molecule in acid or neutral solution if a coordination number of 6 is assumed, are also disregarded. For details see the text.

We propose that OH^-, as a unidentate ligand, plays an essential role in effecting a coulombic interaction with a positively charged pyridinium ring of NAD^+ as illustrated in Fig. 4. As illustrated in this figure, the acidic (410 μM) and alkaline (5.2 μM) asymptotes assumed here are very close to the pH-insensitive values of $K_{EI,O} = 440\ \mu M$, and $K_{EFA,O} = 5\ \mu M$, where I = imidazole and FA = a fatty acid such as caprate. This emphasizes the validity of the older assumption [8] that the E-imidazole-O and E-fatty acid-O complexes are analogous to the acid and alkaline extremes of the EO complex. These analogies can be explained by assuming that the Zn^{2+}-bound H_2O is replaced by imidazole and OH^- is replaced by fatty acid.

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