A Transition State Analogue for Two Pyruvate Metabolizing Enzymes, Lactate Dehydrogenase and Alanine Dehydrogenase†

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ABSTRACT: The synthesis of 5-(2-oxalylethyl)-NADH, a reduced nicotinamide adenine dinucleotide (NADH) derivate with pyruvate covalently attached to the 5 position of the dihydronicotinamide ring over an additional methylene group, has been described previously (Trommer, W. E., Blume, H., and Kapmeyer, H. (1976), *Justus Liebigs Ann. Chem.*, 848). In the presence of lactate dehydrogenase, the dihydropyridine ring of this coenzyme-substrate analogue is oxidized and the carbonyl function of the side chain is reduced to the corresponding L-hydroxy derivate with a maximum velocity of $^1/_{3000}$ of the natural reaction. This reaction is intramolecular as shown by competition experiments with pyruvate. 5-(2-Oxalylethyl)-NADH (pyr-NADH) appears to be a true transition state analogue, proving its postulated structure. Pyr-NADH is highly specific for this enzyme as demonstrated by the facts that (1) D-lactate dehydrogenase does not catalyze the intramolecular redox reaction, although the substrate moiety of pyr-NADH is reduced in the presence of NADH; (2) when tested with malate dehydrogenase, alcohol dehydrogenase, glyceraldehyde phosphate dehydrogenase, glycerate dehydrogenase, and glycerol dehydrogenase pyr-NADH is not even oxidized in the presence of the corresponding substrates. However, a great similarity between the transition states of the reduction of pyruvate catalyzed by lactate dehydrogenase and alanine dehydrogenase could be shown. Alanine dehydrogenase catalyzes the intramolecular redox reaction as well. In the presence of ammonium ions, pyr-NADH is transformed to 5-(3-carboxy-3-aminopropyl)-NAD^+.

Lactate dehydrogenase catalyzes the interconversion of pyruvate and L-lactate in the presence of NADH[1] or NAD^+. A hydride ion is transferred directly from position 4 of the nicotinamide ring to the carbonyl function of pyruvate and vice versa (Fischer et al., 1951). A close steric proximity between the substrate and coenzyme is, therefore, to be expected on the enzyme. This is confirmed by x-ray crystallographic data on abortive ternary complexes of the dogfish muscle isoenzyme (Adams et al., 1973). Covalent attachment of the substrate to the coenzyme with a geometry appropriate for intramolecular hydride transfer should yield transition-state analogues of the enzymatic reaction. Since the equilibrium of the reaction catalyzed by lactate dehydrogenase favors lactate and NAD^+, such an analogue should be composed of pyruvate and the reduced coenzyme when a hydride transfer is to be observed. Recently, pyr-NADH has been synthesized in our laboratory (Figure 1) (Trommer et al., 1976). The substrate, pyruvic acid, is bound via an additional methylene group to position 5 of the nicotinamide ring. The methylene group is necessary for sufficient proximity between the carbonyl function of pyruvate and position 4 of the ring. In the following, we describe the behavior of pyr-NADH in the presence of various dehydrogenases. A preliminary report of some of these findings has been given (Trommer et al., 1975a).

Experimental Procedure

Materials. Lactate dehydrogenase (EC 1.1.1.27) from pig heart and pig muscle, D-lactate dehydrogenase (EC 1.1.1.28) from *Lactobacillus leichmanii*, alcohol dehydrogenase (EC 1.1.1.1) from horse liver and yeast, mitochondrial malate dehydrogenase (EC 1.1.1.37) from pig heart, alanine dehydrogenase (EC 1.4.1.1) from *Bacillus subtilis*, glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle, and NAD^+ glycohydrolase (EC 3.2.2.5) from pig brain were obtained from Boehringer/Mannheim. Glycerate dehydrogenase (EC 1.1.1.29) from spinach leaves and glycerol dehydrogenase (EC 1.1.1.6) from *Aerobacter aerogenes* were purchased from Sigma Chemical Co. NAD^+ and NADH were obtained from Boehringer/Mannheim.

Chemical and Enzymatic Syntheses. The syntheses of 5-(2-oxalylethyl)-NADH and 5-(3-carboxy-3-hydroxypropyl)nicotinamide have been described previously (Trommer et al., 1976).

5-(3-Carboxy-3-hydroxypropyl)-NAD^+ (*lac-NAD^+*) was prepared from NAD^+ and 5-(3-carboxy-3-hydroxypropyl)-nicotinamide by transglycosidation catalyzed by NAD^+ glycohydrolase (pH 7.5; 37 °C; 180 min) (Trommer et al., 1975b; Kaplan and Ciotti, 1956). The protein was denatured by trichloroacetic acid and subsequently centrifuged at 40 000*g*. The supernatant was chromatographed on DEAE-Sephadex A-25 with a linear gradient of 0–0.25 M formic acid. Fractions containing the NAD^+ analogue were identified by the 320-nm absorption of an aliquot after cyanide addition. Lac-NAD^+ was finally isolated after lyophilization and repeated precipitation with acetone, yield 33% based on NAD^+. Anal. Calcd for $C_{25}H_{33}N_7O_{17}P_2 \cdot 2H_2O$ (801.5): C, 37.45; H, 4.62; N, 12.23; P, 7.73. Found: C, 37.23; H, 5.08; N, 12.16; P, 6.90.

5-(3-Carboxy-3-hydroxypropyl)-NADH (*lac-NADH*) was prepared by reduction of lac-NAD with dithionite at 100 °C for 1 min, according to the method of Gutcho and Stewart (1948). The product was purified by chromatography on DEAE-Sephadex A-25 with a linear gradient of 0–0.3 M LiCl. Fractions containing the reduced analogue, as monitored by its 342-nm absorption, were combined, concentrated under reduced pressure, and, finally, precipitated with 2-propanol.

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[1] Abbreviations used are: pyr-NADH, 5-(2-oxalylethyl)-NADH; lac-NADH, 5-(3-carboxyl-3-hydroxypropyl)-NADH; lac-NAD^+, 5-(3-carboxyl-3-hydroxypropyl)-NAD^+; Ala-NAD^+, 5-(3-carboxyl-3-aminopropyl)-NAD^+; ADPRib, adenine diphosphoribose; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; uv, ultraviolet.

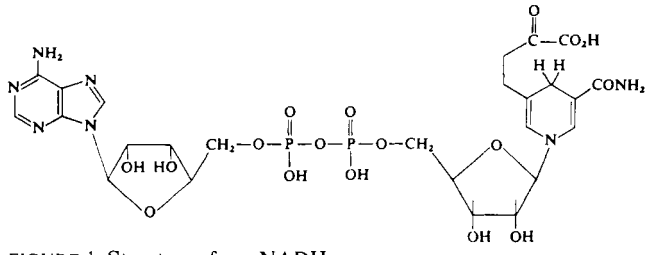

FIGURE 1: Structure of pyr-NADH.

Lac-NADH shows the typical absorption spectrum of reduced NAD^+ analogues with two absorption maxima at 258 nm ($\epsilon = 14.1 \times 10^3$ cm^2/M) and 342 nm ($\epsilon = 6.0 \times 10^3$ cm^2/M).

5-Carboxy-NADH was prepared in the same manner from 5-carboxy-NAD^+ (Trommer et al., 1975b).

Enzymatic Cleavage of NAD^+ Analogues. Five millimoles of the NAD^+ analogue was mixed with 0.1 g of activated NAD^+ glycohydrolase in 20 ml of 0.1 M phosphate buffer (pH 7.5). After incubation for 2 h at 37 °C under shaking, the protein was denatured by trichloroacetic acid and separated by centrifugation. The nicotinamide analogue formed was purified by chromatography on DEAE-Sephadex A-25 with a linear gradient of 0–0.8 M acetic acid, as described previously (Trommer et al., 1976).

Protein Determination. The concentrations of most of the dehydrogenases were determined by their ultraviolet absorption at 280 nm using the following optical densities for solutions containing 1 mg/ml: lactate dehydrogenase, 1.32; alcohol dehydrogenase from horse liver, 0.42; alcohol dehydrogenase from yeast, 1.26; malate dehydrogenase, 0.27; alanine dehydrogenase, 0.64. Alternatively, the biuret reaction (Beisenherz, 1956) was applied using a factor of 8.3 at 546 nm for a final volume of 5 ml in cuvettes with a 2-cm light path.

Enzyme Assays. The crystalline suspensions of the various dehydrogenases were desalted by extensive dialysis against the appropriate buffer. Standard methods were used for measuring the enzymatic activity (Bergmeyer, 1970). NAD^+ glycohydrolase, available as acetone powder, was activated by sonification at 20 kilocycles prior to use (Browne et al., 1971). The specific activity (280 units/mg) was determined as described by Kaplan (1955). All enzyme assays were performed at 25 °C in glass cells with a 1-cm light path. The change in absorbance at 366 nm was monitored by an Eppendorf photometer.

Inhibition experiments of lactate and malate dehydrogenases by pyr-NADH were carried out in a total volume of 0.5 ml of 50 mM phosphate buffer at 25 °C. The reactions were initiated by addition of the enzyme and followed by the decrease of absorbance at 366 nm. Lactate dehydrogenase: the cell contained 0.5 mM pyruvate, 15–50 μM NADH, 0, 0.17, or 0.87 μM inhibitor, and 0.33 μg of enzyme (420 units/mg). Malate dehydrogenase: the cell contained 0.5 mM oxaloacetate, 15–100 μM NADH, 0, 0.09, 0.18, or 0.9 μM inhibitor, and 0.11 μg of enzyme (770 units/mg).

Intramolecular Redox Reaction of Pyr-NADH. For the assay of the redox reaction catalyzed by lactate dehydrogenase, the cell contained in 0.5 ml: 53 mM phosphate (pH 7.2), 0.4 mM pyr-NADH, and 0.7–0.8 mg of the enzyme. When alanine dehydrogense catalyzed the redox reaction, the reaction mixture contained in 0.5 ml: 36.5 mM Tris-HCl (pH 8.0), 0.63 M NH_4Cl, 0.4 mM pyr-NADH, and 0.06–0.07 mg of protein. The concentration of pyr-NADH was determined by its uv absorption using molar extinction coefficients of $\epsilon = 1.4 \times 10^4$ cm^2/M at 260 nm, $\epsilon = 6.0 \times 10^3$ cm^2/M at 342 nm, and $\epsilon = 3.3 \times 10^3$ cm^2/M at 366 nm.

TABLE I: R_f Values of NAD Analogues.

Compound	R_f Value in System A	B	C	Electrophoretic Mobility ($cm^2/Vs \times 10^6$)
Pyr-nicotinamide	0.44	0.77	0.51	15.7
Lac-nicotinamide	0.47	0.70	0.49	10.9
Pyr-NAD^+	0.23	0.62	0.24	9.3
Pyr-NADH	0.32	0.75	0.36	14.1
Lac-NAD^+	0.23	0.62	0.23	
Lac-NADH	0.27	0.75	0.33	
NAD^+	0.31	0.55	0.20	4.2
NADH	0.32	0.74	0.38	7.7

Substrate-Analogous Reduction of Pyr-NADH. The reaction mixture contained in 0.5 ml: 53 mM phosphate (pH 7.2), 0.5 mM pyr-NADH, 0.4 mM NADH, and 0.5 mg of D-lactate dehydrogenase.

Substrate Analogous Oxidation of 5-(3-Carboxy-3-hydroxypropyl)nicotinamide. The reaction was performed in a final volume of 1 ml containing 0.2 M hydrazine, 0.5 M glycine (pH 9.5), 0.26 mM EDTA, 2.4 mM NAD^+, 0.6 mM 5-(3-carboxy-3-hydroxypropyl)nicotinamide, and 0.7–0.8 mg of lactate dehydrogenase.

Thin-layer chromatography was carried out on precoated silica gel F-254 plates from Merck/Darmstadt. The following systems were used. System A: isobutyric acid–concentrated ammonia–water (66:1:33), pH 4.2; system B: ammonium acetate–95% ethanol (1:1), pH 6.0; system C: propanol–concentrated ammonia–water (20:12:3); system D: pyridine–acetic acid–water–butanol (40:14:25:68), pH 6.4. (See Table I for R_f values and electrophoretic mobilities.)

Electrophoresis was carried out on Whatman no. 3MM paper in 0.1 M ammonium acetate buffer, pH 4.6, at 25 V/cm.

Fluorescence titrations were carried out with a Perkin-Elmer spectrofluorimeter Model MPF-2A. To determine the binding constant of 5-carboxy-NADH to lactate dehydrogenase, malate dehydrogenase, and alcohol dehydrogenase, 0.4 mg/ml of protein was titrated with 5-μl samples of 0.6 mM 5-carboxy-NADH up to a final concentration of 0.04 mM. Excitation and emission settings were 360 and 440 nm, respectively.

For the determination of the ternary-complex binding capacity, the change of the fluorescence intensity at 440 nm was measured upon addition of 25 μM oxamate (for the complex with lactate dehydrogenase) or 0.5 M acetamide (for the complex with alcohol dehydrogenase) to a mixture containing 0.4 mg/ml of enzyme and 10 μM 5-carboxy-NADH.

Results

Redox Reaction Catalyzed by Lactate Dehydrogenase. Pyr-NADH was shown to be stable in neutral buffer solutions; i.e., no decomposition occurred in 67 mM phosphate buffer during 10 h. However, upon addition of lactate dehydrogenase from pig heart or pig muscle, marked changes in its absorption spectrum were observed. The maximum at 340 nm decreased, while that at 260 nm increased. In Figure 2, the time course of such a reaction is shown. From 20 to 25% of the original optical density at 340 nm remained unchanged. Consecutive addition of cyanide or sulfite ions led to a new absorption at 327 or 323 nm, typical for NAD^+ derivatives. The product could be identified as lac-NAD^+, as demonstrated by the following experiments after chromatographical purification. It coeluated with the synthetic lac-NAD^+ from DEAE-Sephadex

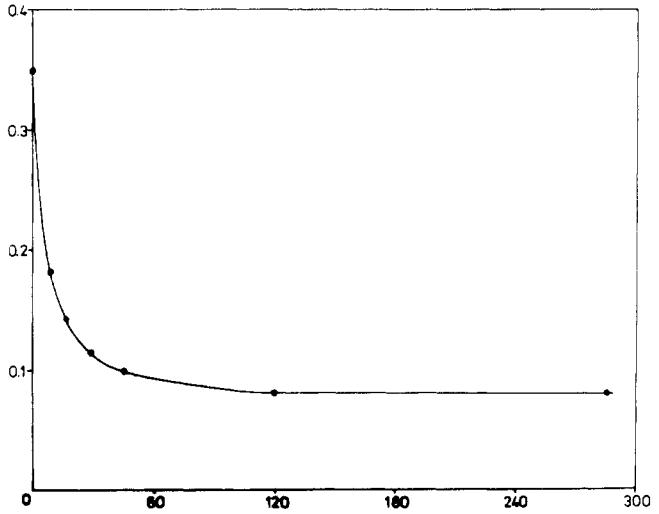

FIGURE 2: Time course of the decrease of the 340-nm absorption of 60 μM pyr-NADH upon addition of 20 μM lactate dehydrogenase.

TABLE II: Inhibition of Various Dehydrogenases by Pyr-NADH and 5-Carboxy-NADH.[a]

	Pyr-NADH		5-Carboxy-NADH	
	0.044 mM	0.44 mM	0.044 mM	0.44 mM
Lactate dehydrogenase	89	100	0	37
D-Lactate dehydrogenase	36	85	11	31
Malate dehydrogenase	95	100	7	62
Alanine dehydrogenase	6	17	6	23
Alcohol dehydrogenase (horse liver)	0	6	7	52
Glycerate dehydrogenase	11	46		
Glyceraldehyde-phosphate dehydrogenase	5	11		
Glycerol dehydrogenase	10	28		

[a] NADH derivatives to a final concentration, as indicated, were added to the assay mixtures containing 0.44 mM NADH. Values are given as percentage of inhibition.

A-25 and they had identical mobilities and R_f values on electrophoresis and thin-layer chromatography (systems A, B, and C). Hydrolysis by NAD^+ glycohydrolase yielded ADPRib and 5-(3-carboxy-3-hydroxypropyl)nicotinamide, as revealed by thin-layer chromatography (systems A, B, and C). To demonstrate the stereospecificity of the redox reaction, this 5-(3-carboxy-3-hydroxypropyl)nicotinamide was isolated as described under Experimental Procedures and subjected to a substrate-analogous reaction with NAD^+ and D- and L-specific lactate dehydrogenase. Oxidation is observed only in the presence of L-specific lactate dehydrogenase, whereas the synthetic racemic compound is partially oxidized by both enzymes.

A double-reciprocal plot of the steady-state kinetic experiment (Lineweaver and Burk, 1934) yielded a K_M value for pyr-NADH, at pH 7.2, of 2.9×10^{-4} M, only about tenfold greater than that of NADH ($K_M = 2.1 \times 10^{-5}$ M), although the maximum velocity is about $1/3000$ of that of NADH (pyr-NADH: $V_{max} = 0.162$ units/mg; NADH: $V_{max} = 418$ units/mg).

No change in the reaction rate was found upon addition of pyruvate up to 5 mM. In the presence of NADH, an inhibition of 50% occurred when the reaction mixture contained NADH in the same concentration as pyr-NADH.

Substrate-Analogous Reduction with D-Lactate Dehydrogenase. D-Lactate dehydrogenase from *Lactobacillus leichmanii* did not catalyze the redox reaction of pyr-NADH. When NADH was added, a reaction was noticed by a decrease of the absorption maximum at 340 nm. The product was identified as lac-NADH by comparison with the synthetic compound by thin-layer chromatography (system A, B, and C) and anion exchange chromatography. In the presence of pyruvate no coenzymatic activity of pyr-NADH could be observed.

Redox Reaction Catalyzed by Alanine Dehydrogenase. A redox reaction of pyr-NADH could also be obtained with alanine dehydrogenase. Since alanine dehyrogenase catalyzes the reductive amination of pyruvate, the redox reaction took place only in presence of ammonium ions. The new NAD^+ analogue differed in its R_f value from lac-NAD^+. After purification by anion-exchange chromatography (DEAE-Sephadex A-25, linear gradient of 0–0.2 M formic acid) and subsequent hydrolysis by NAD^+ glycohydrolase, two products could be demonstrated by thin-layer chromatography, ADPRib ($R_f = 0.07$ in system D) and a nicotinamide derivative ($R_f = 0.27$), having a primary amino function as shown by its reaction with ninhydrine. Separation from excess ammonium ions had been achieved by chromatography on silica in system D. The product of this redox reaction, therefore, must have been Ala-NAD^+.

The K_M value for pyr-NADH at pH 8.0, as determined by a double-reciprocal plot of the steady-state kinetic experiment, was found to be the same as for NADH ($K_M = 2.25 \times 10^{-5}$ M) (Yoshida and Freese, 1964). Again, the maximum velocity was only about $1/1500$ of that of NADH (pyr-NADH: $V_{max} = 0.264$ units/mg; NADH: $V_{max} = 378$ units/mg).

With 10 mM pyruvate there was only a 12% inhibition, while a 75% reduced reaction velocity occurred in the presence of equal amounts of NADH and pyr-NADH.

Enzymatic Assays with NAD-Dependent Dehydrogenases. Enzymatic assays with other NAD-dependent dehydrogenases, such as malate hydrogenase, alcohol dehydrogenase, glyceraldehyde-phosphate dehydrogenase, glycerate dehydrogenase, and glycerol dehydrogenase, were studied too. No redox reaction of pyr-NADH could be achieved. Even in the presence of the corresponding substrates, no coenzymatic activity was detected.

Inhibition Studies. The effects of pyr-NADH and 5-carboxy-NADH upon the catalytic activity of the various dehydrogenases were investigated (reaction NADH $\rightarrow$ NAD^+). Such studies were performed by adding a suitable amount of the NADH derivative to the enzymatic assay mixtures (standard concentrations) and observing the inhibitory effect on the rate of reaction. Table II summarizes the percentage of inhibition that was observed when one-tenth or equal amounts of the analogue and NADH were present in the mixtures. As can be seen from the table, pyr-NADH was a highly effective inhibitor of malate and lactate dehydrogenases only. The other dehydrogenases showed only a slight inhibition by both NADH derivatives. Investigation into the nature of the strong inhibition of lactate and malate dehydrogenases, as described under Experimental Procedures, revealed a purely competitive inhibition of pyr-NADH with respect to NADH when plotted according to the method of Lineweaver and Burk (1934). Lactate dehydrogenase: $K_I = 1.35 \times 10^{-7}$ M; malate dehydrogenase: $K_I = 8.9 \times 10^{-8}$ M.

Binding of 5-Carboxy-NADH to Dehydrogenases. No oxidation of 5-carboxy-NADH in the presence of various dehydrogenases and the corresponding substrates has been observed

though the formation of a binary enzyme: 5-carboxy-NADH complex could be demonstrated by fluorescence measurements. Addition of 5-carboxy-NADH to solutions of lactate dehydrogenase, malate dehydrogenase, or alcohol dehydrogenase resulted in an enhancement of its fluorescence around 460 nm. From titration experiments, the equilibrium constant for the dissociation of the binary complex was calculated. Figure 3 shows a computer fit to the experimental data for lactate and malate dehydrogenases by an iterative procedure according to the method of Engel (1974). In the case of alcohol dehydrogenase from horse liver, a Scatchard plot (Scatchard, 1948) showed no linear relationship. There are at least two different sets of binding sites.

No formation of a ternary lactate dehydrogenase–5-carboxy-NADH–oxamate complex took place, as demonstrated by the lack of fluorescence quenching upon addition of the substrate analogue. In a similar experiment with alcohol dehydrogenase, addition of acetamide to a mixture of alcohol dehydrogenase and 5-carboxy-NADH, no fluorescence enhancement was observed. In the ternary complex with NADH, the fluorescence is enhanced under these conditions (Holbrook and Gutfreund 1973).

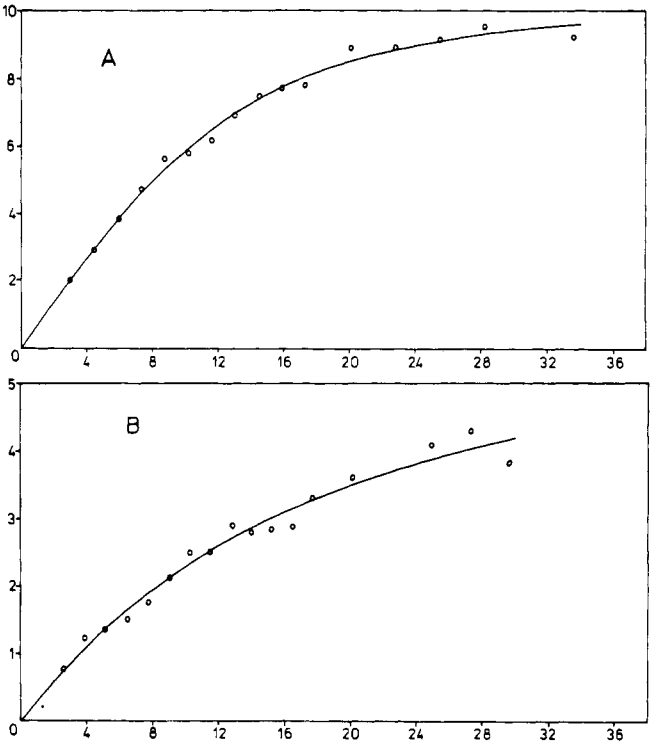

FIGURE 3: Computer-drawn dissociation curve of the fluorescence titration data of 5-carboxy-NADH binding to (A) lactate dehydrogenase, K_D = 2.57 × 10^{-6} M, and (B) malate dehydrogenase, K_D = 1.02 × 10^{-5} M.

Discussion

Pyr-NADH is the first coenzyme substrate analogue of a dehydrogenase in which an enzyme-catalyzed hydride transfer takes place. In the presence of lactate dehydrogenase, it is converted to lac-NAD^+, again a coenzyme–substrate analogue but now with the reduced substrate and the oxidized form of the coenzyme covalently attached to one another. This reaction proceeds to about 80% completion only, although judging from the equilibrium constant of the pyruvate reduction by NADH a nearly complete ring oxidation should be possible. The alkyl substituent alters the redox potential, as determined by cyanide addition to pyr-NAD^+ (Wallenfels and Dieckmann, 1959), by 15 mV even more in favor of lactate and NAD^+. A likely explanation is a contamination with the α isomer of pyr-NADH arising from the chemical reduction and the subsequent chromatography. When NAD^+ is subjected to the same conditions, about 17% cannot be reoxidized enzymatically. In our case, a separation is not possible due to the highly acidic side chain determining the elution behavior from ion-exchange resins. Lactate dehydrogenase catalyzes the transfer of the H_R proton of the dihydronicotinamide ring to the carbonyl function of pyruvate under formation of L-lactate (Levy and Vennesland, 1957). A molecular model of pyr-NADH reveals that, in case of an intramolecular hydride shift, two possible products may arise, L-lac-NAD^+ under shift of H_R and D-lac-NAD^+ under shift of H_S. The two alternative reactions would require an orientation of the side chain in which the carboxyl group interferes with the pyridine ring. The substrate-analogous oxidation of 5-(3-carboxy-3-hydroxypropyl)nicotinamide, obtained from lac-NAD^+ by enzymatic cleavage, shows the L isomer of lac-NAD^+ to be the only product. The oxidation is catalyzed exclusively by the L-specific enzyme. No oxidation is observed with D-lactate dehydrogenase, whereas the synthetic racemic compound is partially oxidized by both enzymes. Above, we have claimed the redox reaction to be intramolecular. An intermolecular mechanism is conceivable, as well, one molecule of pyr-NADH acting as coenzyme and a second one as substrate. It is known that 5-substituted NAD^+ derivatives, 5-amino-, 5-methyl- (Walter and Kaplan, 1963), and 5-carboxy-NAD^+, do not act as coenzyme. 5-Carboxy-NAD^+ was shown to be a competitive inhibitor of NAD^+ in the lactate dehydrogenase system (Trommer et al., 1975b); however, it does not form the ternary lactate dehydrogenase–coenzyme–sulfite complex (Pfleiderer and Jeckel, 1957). In this investigation, we show by fluorescence titration experiments that 5-carboxy-NADH is bound nearly as tightly to lactate dehydrogenase as NADH itself. Again, no ternary complex (lactate dehydrogenase–5-carboxy-NADH–oxamate) is formed. Oxamate does not quench the fluorescence of the lactate–5-carboxy-NADH complex. Consequently, 5-carboxy-NADH is coenzymatically not active. It clearly follows that five substituents of the nicotinamide ring interfere with substrate binding. Therefore, it seems unlikely that the pyruvate moiety of pyr-NADH could act as substrate when a second molecule of pyr-NADH is bound to the active center. Moreover, there is an even stronger argument for an intramolecular mechanism. In an intermolecular mechanism, the rate of the redox reaction should depend on the concentration of added pyruvate. Several investigators have studied the reduction rates of pyruvate analogues by lactate dehydrogenase (Czok and Bücher, 1960). The turnover is generally low, particularly when the analogue carries bulky substituents at C-3; e.g., *o*-nitrophenyl pyruvate exhibits a maximum velocity of $1/2000$ of that of pyruvate (Holbrook and Stinson 1973). A similar value is obtained when pyr-nicotinamide is used as substrate (W. E. Trommer et al., unpublished). However, the rate of formation of lac-NAD^+ is fully independent of the pyruvate concentration. In the presence of NADH, the turnover is even reduced. In an intermolecular reaction an enhancement would be expected. Since the Michaelis constant of NADH is ten times smaller than that of pyr-NADH (NADH: K_M = 0.21 × 10^{-4} M; Pyr-NADH: K_M = 2.94 × 10^{-4} M), it competes successfully with the analogue for the binding site. Although the affinity of pyr-NADH to lactate dehydrogenase is nearly as high as that of NADH itself, the turnover is rather low (NADH: V_{max} = 418 units/mg; pyr-NADH: V_{max} = 0.162 units/mg). As pointed out above, pyruvate analogues are reduced rather

slowly by lactate dehydrogenase. This has been attributed to the fact that the conformational change to the activated ternary complex is hindered (Holbrook et al., 1975). The methylene spacer in pyr-NADH might hinder this conformational change as well. As shown above, there is limited space around the 5 position of the nicotinamide ring in the ternary complex; i.e., even small substituents inhibit substrate binding. An alternative explanation for the low turnover is provided by the fact that the normal ternary product complex dissociates with compulsory order. Ternary complexes exhibit considerably smaller apparent dissociation constants of the coenzyme than the corresponding binary complexes. In pyr-NADH a direct dissociation from a "ternary" complex is required. In contrast to the results with the L-specific lactate dehydrogenase, no hydride transfer was observed in the presence of the D-specific enzyme. This result is not surprising when one considers that in D-lactate dehydrogenase the same H_R proton of the dihydropyridine ring is transferred to the substrate as in the L-specific enzyme (Dennis and Kaplan, 1960; Levy and Vennesland, 1957). This may imply that the substrate has to point into the opposite direction relative to the nicotinamide ring to allow for the formation of the D isomer. Pyr-NADH, therefore, would not represent the transition state of the pyruvate reduction by D-lactate dehydrogenase. Long and Kaplan have recently proposed an alternative model allowing for the formation of either D- or L-lactate by two closely related enzymes, in which the substrate is perpendicular to the plane of the ring (Long and Kaplan 1973). However, if that is the actual arrangement, it would seem unlikely that the L-specific enzyme could catalyze the redox reaction. The formation of lac-NADH in the presence of NADH results from an intermolecular reaction in which the pyruvate moiety of pyr-NADH acts as substrate. This would not require the simultaneous binding of the two coenzyme molecules to the enzyme. An arrangement could be envisaged in which NADH is bound to the coenzyme binding site, and the extended substrate chain of pyr-NADH reaches into the active center while most of the coenzyme part of this analogue is still in the surrounding solution.

Lactate dehydrogenase is not the only NAD-dependent dehydrogenase catalyzing the reduction of pyruvate. In the presence of ammonium ions, a reductive amination to L-alanine is catalyzed by alanine dehydrogenase. This enzyme catalyzes the hydride transfer in pyr-NADH in the presence of ammonium ions to ala-NAD^+. The redox reaction follows an intramolecular mechanism, because NADH again inhibits this reaction and pyruvate does not enhance the fairly low turnover (NADH: V_{max} = 378 units/mg; pyr-NADH: V_{max} = 0.264 units/mg). This implies a great similarity in the arrangement of pyruvate and NADH in the transition states of the conversion to L-alanine and NAD^+ by alanine dehydrogenase and to lactate and NAD^+ by lactate dehydrogenase.

From these results one could predict alanine dehydrogenase to be A-side specific with respect to NADH, which, to our knowledge, is not yet known. Pyr-NADH is highly specific for pyruvate metabolizing enzymes. Even when the substrates do not differ greatly from pyruvate, as 3-hydroxypyruvate, in glycerate dehydrogenase, or oxaloacetate in malate dehydrogenase, no redox reaction takes place. From the inhibition experiments, no clearcut results could be obtained. As shown in Table II, enzymes with acidic substrates are generally inhibited more strongly. Interesting is the very high inhibition of malate dehydrogenase. Pyr-NADH is a competitive inhibitor with respect to NADH, exhibiting an inhibition constant of $K_I = 0.89 \times 10^{-7}$ M. In spite of the intramolecular redox reaction, the pyr-NADH inhibition of the lactate dehydrogenase catalyzed reduction of pyruvate with NADH as coenzyme was studied. Because of the very low turnover, the intramolecular reaction could be neglected. Pyr-NADH is a competitive inhibitor, exhibiting very high affinity for the enzyme ($K_I = 1.35 \times 10^{-7}$ M). To our knowledge, this is the smallest K_I for a NADH derivative found so far (Everse et al., 1971). As can be seen from Table I, 5-carboxy-NADH is a much weaker inhibitor, demonstrating the importance of the pyruvate side chain of pyr-NADH for its binding to lactate dehydrogenase. Pyr-NADH appears to be a true transition state analogue of the interconversion of pyruvate and lactate catalyzed by this enzyme.

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