Combined Coenzyme–Substrate Analogues of Various Dehydrogenases. Synthesis of (3*S*)- and (3*R*)-5-(3-Carboxy-3-hydroxypropyl)nicotinamide Adenine Dinucleotide and Their Interaction with (*S*)- and (*R*)-Lactate-Specific Dehydrogenases†

Ulrich Grau, Hannelore Kapmeyer,‡ and Wolfgang E. Trommer*

ABSTRACT: Two diastereomeric nicotinamide adenine dinucleotide (NAD^+) derivatives were synthesized in which the substrates of (*S*)- and (*R*)-lactate-specific dehydrogenases are covalently attached via a methylene spacer at position 5 of the nicotinamide ring. The corresponding nicotinamide derivatives were obtained stereospecifically by enzymatic reduction of 5-(2-oxalylethyl)nicotinamide. (3*S*)-5-(3-Carboxy-3-hydroxypropyl)-NAD^+ undergoes an intramolecular hydride transfer in the presence of pig heart lactate dehydrogenase, forming the corresponding coenzyme–substrate analogue composed of pyruvate and NADH. No cross-reaction products resulting from an intermolecular reaction are observed. Two (*R*)-lactate specific dehydrogenases, however, do not catalyze a similar reaction in either one of the two diastereomers. A possible arrangement of the substrates in the active centers of these enzymes is proposed. 5-Methyl-NAD^+ and 5-methyl-NADH are active coenzymes of pig heart lactate dehydrogenase in contrast to reports in the literature. (*S*)-Lactate binds to this enzyme in the absence of coenzyme, exhibiting a dissociation constant of 11 mM.

Two forms of lactate dehydrogenase have been found with different specificities. The enzymes from all vertebrates and those from plants and some microorganisms form (*S*)-lactate (Oba et al., 1977; Yoshida and Freese, 1965). (*R*)-Lactate formation has been described primarily in microorganisms (Tarmy and Kaplan, 1968) but also in several invertebrates as well (Long and Kaplan, 1973a). Whereas the *S*-specific enzyme has been studied exceedingly well, including X-ray analysis (Holbrook et al., 1975), little is known about the (*R*)-lactate dehydrogenases. The difference in the relative arrangement of coenzymes and substrates in the active centers of the two enzymes has been the matter of considerable speculation (Long and Kaplan, 1973b; Dennis and Kaplan, 1960). Both types have been shown to transfer the same *pro-R* hydrogen from the dihydropyridine ring to pyruvate, with specific formation of the anomeric lactates (Levy and Vennesland, 1957; Dennis and Kaplan, 1959).

Recently, we have described a combined coenzyme–substrate analogue for lactate dehydrogenase, pyr-NADH,[1] in which pyruvic acid is covalently bound via a methylene spacer to position 5 of the dihydronicotinamide ring (Kapmeyer et al., 1976). Addition of (*S*)-lactate-specific enzymes causes an intramolecular hydride transfer that forms the corresponding analogue composed of NAD^+ and (*S*)-lactate. Pyr-NADH should represent the relative arrangement of coenzyme and substrate in these enzymes rather well, as demonstrated by its very tight binding. With the (*R*)-lactate-specific enzymes, however, no hydride transfer is observed. We have now independently prepared (*S*)- and (*R*)-lac-NAD^+ (Figure 1) and have studied their interaction with both enzymes in order to gain some insight in the different geometries of their active centers.

Experimental Procedure

Materials. Pig heart lactate dehydrogenases (EC 1.1.1.27), (*R*)-lactate dehydrogenases from *Lactobacillus leichmannii* (EC 1.1.1.28), and NAD^+ glycohydrolase from pig brain (EC 3.2.2.5) were obtained from Boehringer/Mannheim. (*R*)-Lactate dehydrogenase from *Limulus* was a generous gift from G. L. Long. NAD^+, NADH, and (*S*)- and (*R*)-lactate were purchased from Boehringer/Mannheim, and (*S*)-[3-^{14}C]-lactate (8 Ci/mol) was from New England Nuclear.

Chemical and Enzymatic Syntheses. (3*S*)-5-(3-Carboxy-3-hydroxypropyl)nicotinamide[(*S*)-Lac-nicotinamide]. Pyr-nicotinamide (342 mg, 1.54 mmol) (Trommer et al., 1976) was dissolved in 150 mL of 40 mM phosphate buffer (pH 7.2) containing 0.1 M ammonium bicarbonate. After the addition of 9 mL of pig heart lactate dehydrogenase (8 mg/mL, freshly dialyzed against phosphate buffer), a solution of 635 mg of NADH (0.9 mmol) in 8 mL of water was added in small portions under gentle stirring at 25 °C. The reaction was followed by the decrease in its 340-nm absorption and was carefully kept at pH 7.2 by the addition of dilute acetic acid. After complete oxidation of NADH, the protein was denatured by the addition of trichloroacetic acid and separated by centrifugation at 40 000*g*. The supernatant is lyophilized for several days to remove any ammonium bicarbonate prior to the purification by anion-exchange chromatography of DEAE-Sephadex A 25 (acetate form) with a linear gradient of 0–1 M acetic acid. (*S*)-Lac-nicotinamide eluted at 0.4 M acetic acid, whereas unreacted pyr-nicotinamide may be recovered at 0.8 M. Further purification is achieved by repeated precipitation from an aqueous solution with acetone: yield 183 mg (90% based on NADH). Anal. Calcd for $C_{10}H_{12}N_2O_4$ (224.22): C, 53.57; H,

† From the Institut für Organische Chemie, Biochemie und Isotopenforschung der Universität Stuttgart, 7000 Stuttgart 80, West Germany. *Received March 23, 1978.*

‡ Present address: Department of Chemistry, University of California at San Diego, La Jolla, Calif.

[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; lac-NAD^+, 5-(3-carboxy-3-hydroxypropyl)-NAD^+; pyr-NAD^+, 5-(2-oxalylethyl)-NAD^+; pyr-NADH, 5-(2-oxalylethyl)-NADH; lac-nicotinamide, 5-(3-carboxy-3-hydroxypropyl)nicotinamide; pyr-nicotinamide, 5-(2-oxalylethyl)nicotinamide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; ORD, optical rotation dispersion. Enzymes used: (*S*)-lactate dehydrogenase, L-lactate:NAD^+ oxidoreductase (EC 1.1.1.27); (*R*)-lactate dehydrogenase, D-lactate:NAD^+ oxidoreductase (EC 1.1.1.28).

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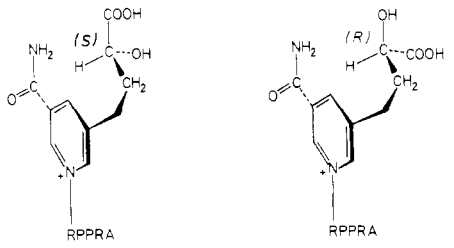

FIGURE 1: Structures of (*S*)- and (*R*)-lac-NAD$^+$.

5.40; N, 12.49. Found: C, 53.33; H, 5.51; N, 12.35. R_f value in system A, 0.46; B, 0.73; C, 0.53; $[\alpha]^{20}_D$ +3.40° (*c* 0.59) in water.

(3*R*)-5-(3-Carboxy-3-hydroxypropyl)nicotinamide [(*R*)-Lac-nicotinamide]. The procedure was the same as described for the *R* stereoisomer, except that (*R*)-lactate dehydrogenase from *Lactobacillus leichmannii* was used: yield 91 mg from 170 mg of pyr-nicotinamide (90% based on NADH). Anal. Calcd for $C_{10}H_{12}N_2O_4$ (224.22): C, 53.57; H, 5.40; N, 12.49. Found: C, 53.61; H, 5.49; N, 12.17. R_f value in system A, 0.46; B, 0.73; C, 0.53; $[\alpha]^{20}_D$ −3.40° (*c* 0.59) in water.

(3*S*)- and (3*R*)-5-(3-Carboxy-3-hydroxypropyl)-NAD$^+$ [(*S*)- and (*R*)-Lac-NAD$^+$]. The corresponding nicotinamide derivative (75 mg, 0.33 mmol) and NAD$^+$ (93 mg, 0.14 mmol) were dissolved in 7 mL of water, neutralized with KOH, and brought to a final volume of 15 mL with 1 M phosphate buffer (pH 7.2) after the addition of 490 mg of NAD$^+$ glycohydrolase (acetone powder, freshly activated by sonification at 20 kilocycles) (Trommer et al., 1975; Kaplan and Ciotti, 1956). The mixture was stirred gently at 37 °C for 3 h, treated with trichloroacetic acid, centrifuged at 40 000*g*, and neutralized. Purification was achieved by chromatography on DEAE-Sephadex A25 (formiate form) with a linear gradient of 0–0.3 M formic acid. Lac-NAD$^+$ eluted with 0.3 M formic acid. After lyophilization, repeated precipitation from an aqueous solution with ethanol yielded 32 mg (30% based on NAD$^+$) of the *S* diastereomer and 25 mg (23%) of the *R* diastereomer.

S Diastereomer. Anal. Calcd for $C_{25}H_{37}N_7O_{17}P_2 \cdot 2H_2O$ (801.56): C, 37.46; H, 4.65; N, 12.23. Found: C, 37.53; H, 5.10; N, 12.35. R_f value in system A, 0.15; B, 0.57; C, 0.31.

R Diastereomer. Anal. Calcd for $C_{25}H_{37}N_7O_{17}P_2$ (765.55): C, 39.22; H, 4.35; N, 12.81; P, 8.09. Found: C, 39.16; H, 4.52; N, 12.89; P, 7.99. R_f value in system A, 0.15; B, 0.57; C, 0.31.

5-Methyl-NAD$^+$ and 5-Methyl-NADH. Both analogues were prepared as originally described by Kaplan and coworkers (Walter and Kaplan, 1963). 5-Methylnicotinamide was obtained by hydrolysis of 3-cyano-5-methylpyridine with alkaline hydrogen peroxide and purified by sublimation.

Protein Determination. The concentrations of the lactate dehydrogenases were determined by their ultraviolet absorption at 280 nm using the following optical densities for solutions containing 1 mg/mL: pig heart, 1.34 (Holbrook et al., 1975); *Lactobacillus leichmannii*, 0.63; *Limulus polyphemus*, 0.73 (Long and Kaplan, 1973a).

Enzyme Assays. The crystalline suspensions of the various dehydrogenases were desalted by extensive dialysis against phosphate buffer (pH 7.2) (for the *R*-specific dehydrogenases containing 14 mM β-mercaptoethanol and 1 mM EDTA). Standard methods were used for measuring the enzymatic activity of the pig heart and *Lactobacillus* enzyme (Bergmeyer, 1970). The *Limulus* enzyme was assayed according to the method of Long and Kaplan (1973a).

Inhibition Experiments with (R)- and (S)-Lac-NAD$^+$. All inhibition experiments with (*R*)- and (*S*)-lactate dehydrogenase were carried out at 25 °C in a total volume of 0.4 mL in 1-cm cuvettes in 1 M glycine buffer (pH 9.5) containing 0.4 M hydrazine. (*S*)- or (*R*)-lactate concentration was 35 mM; NAD$^+$ was varied from 0.04 to 0.3 mM. The reactions were started by the addition of 0.65 μg of the pig heart enzyme (380 units/mg) or 1.2 μg of the *Lactobacillus* enzyme (300 units/mg) and followed by the increase in the 366-nm absorption by means of an Eppendorf photometer. The inhibitor concentrations were 0, 1, and 2 mM in the case of the *S* diastereomer and 0, 0.53, 1.1, and 2.1 mM in the case of the *R* diastereomer. Inhibition constants were evaluated according to Dixon (1953).

Intramolecular Redox Reaction in (S)-Lac-NAD$^+$. The reaction was carried out at 25 °C in 1-cm cuvettes in a total volume of 0.4 mL in 1 M glycine buffer (pH 9.5) containing 0.4 M hydrazine. (*S*)-Lac-NAD$^+$ was varied from 0.16 to 6.4 mM. The hydride transfer after the addition of 0.14 mg of pig heart lactate dehydrogenase (380 units/mg) was followed by the increase of the 366-nm absorption using a molar extinction coefficient of 3.3×10^3 cm^2/mmol (Kapmeyer et al., 1976). The product of this reaction was isolated by ion-exchange chromatography on DEAE-Sephadex A25, formate form, using a linear gradient of 0–0.8 M sodium formate. It was eluted at 0.6 M sodium formate, as was a sample of pyr-NADH which had been treated earlier with glycine–hydrazine buffer (pH 9.5). To assure identical experimental conditions, these runs were carried out simultaneously in parallel columns. The inhibition of the redox reaction by (*S*)-lactate was studied under similar conditions in the presence of 0, 0.3, 0.6, and 1.2 mM inhibitor by variation of (*S*)-lac-NAD$^+$ from 0.4 to 3.2 mM. The inhibition by 1.3 mM NAD$^+$ was studied at 0.7 and 1.4 mM (*S*)-lac-NAD$^+$.

Substrate Analogous Reaction of Pyr-nicotinamide. The reactions were carried out at 25 °C in 67 mM phosphate buffer (pH 7.2) with 0.15 mM NADH and 0.09–0.9 mM pyr-nicotinamide in the case of the *Lactobacillus* enzyme (0.1 mg, 300 units/mg), in 0.4-mL total volume. For the pig heart enzyme (90 μg, 380 units/mg), pyr-nicotinamide was varied from 0.4 to 4 mM. Kinetic constants were evaluated according to the method of Cornish-Bowden (1974).

Coenzyme Analogous Reactions of 5-Methyl-NAD$^+$ and 5-Methyl-NADH. For the reduced coenzyme analogue, the reactions were carried out in 67 mM phosphate buffer (pH 7.2) at 0.55 mM pyruvate and 6–60 μM 5-methyl-NADH and were started with 52 μg of (*S*)-lactate dehydrogenase (380 units/mg). 3-Methyl-NAD$^+$ was varied from 0.28 to 5.6 mM in 1 M glycine buffer (pH 9.5) containing 0.4 M hydrazine and 37 mM (*S*)-lactate. In this case, 90 μg of the enzyme was added. The method of Cornish-Bowden (1974) was applied for evaluation of kinetic constants.

Thin-Layer Chromatography. Thin-layer chromatography was carried out on precoated silica F-254 plates from Merck/Darmstadt in the following systems: A, isobutyric acid–concentrated ammonia–water (66:1:33); B, 1 M aqueous ammonium acetate–95% ethanol (1:1); C, 1-propanol–concentrated ammonia–water (20:12:3).

Determination of the Molar Rotation and ORD Spectra. ORD spectra were recorded on a Cary 60 spectropolarimeter. Molar rotations were calculated according to the following relationship:

$$\Phi = \frac{100\alpha}{cd}$$

Binding of (S)-[3-^{14}C]Lactate to Pig Heart Lactate Dehydrogenase. The ammonium sulfate suspension of the enzyme was centrifuged at 40 000*g*, dissolved in 67 mM phosphate

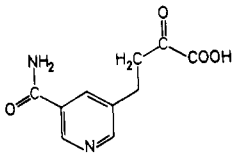

FIGURE 2: Structure of pyr-nicotinamide.

TABLE I: Kinetic Constants of Various Coenzyme and Substrate Analogues of (*S*)- and (*R*)-Lactate Dehydrogenases.

	(*S*)-lactate dehydrogenase[e]		(*R*)-lactate dehydrogenase[f]	
	K_m (mM)	V (units/mg)	K_m (mM)	V (units/mg)
pyr-nicotinamide[a]	0.77	0.088	1.1	0.5
5-methyl-NAD$^+$[b]	1.9	0.043		
5-methyl-NADH[c]	0.017	0.11		
(*S*)-lac-NAD$^+$ intramol	2.3	0.025		
NAD$^+$[b]	0.050	80	2.1	74
NADH[c]	0.021	420		
pyruvate[a]	0.060	420	0.74	720
(*S*)-lactate[d]	3.3	64		

[a] Under saturating conditions of NADH. [b] Under saturating conditions of lactate. [c] Under saturating conditions of pyruvate. [d] Under saturating conditions of NAD$^+$. [e] From pig heart. [f] From *L. leichmannii*.

buffer (pH 7.2), and chromatographed on Sephadex G-25 containing 4 mg/mL finely powdered charcoal (Norit A from Serva/Heidelberg, washed with hydrochloric acid), and then dialyzed against 0.1 M Tris buffer (pH 7.0). Equilibrium dialysis was carried out at 25 °C in a Dianorm apparatus from Diachema AG/Zürich with Spectrapor membranes Type 2 from Spectrum Medical Ind./Los Angeles, equipped with 1-mL half cells containing 0.1 M each of Tris and NaCl in a total volume of 0.8 mL. (*S*)-Lactate was varied from 0.3 to 20 mM at a protein concentration of 0.14 mM (based on a molecular weight of 144 000). The concentration of the ^{14}C-labeled lactate (8 Ci/mol) was determined by means of a liquid scintillation counter (Betaszint 5000) from Labor Professor Berthold/Wildbad.

Results

Substrate Analogous Reduction of Pyr-nicotinamide. Pig heart lactate dehydrogenase was found to catalyze complete conversion of pyr-nicotinamide (Figure 2) by NADH to optically active lac-nicotinamide. The sign of the specific rotation ($[\alpha]^{20}_D$ +3.40°) corresponds to (*S*)-lactate. Given the known stereospecificity of this enzyme and considering the fact that its enantiomer can be prepared in an analogous reaction using an (*R*)-lactate-specific enzyme (see below), the *S* configuration may safely be assigned. The K_m value for pyr-nicotinamide in this reaction is ten times higher then found for pyruvate itself. The maximum velocity reaches only 0.02% of its value in the natural system (Table I). In an analogous reaction, the (*R*)-lactate-specific dehydrogenase from *Lactobacillus leichmannii* catalyzes the formation of (*R*)-lac-nicotinamide from pyr-nicotinamide and NADH. (*R*)- and (*S*)-lac-nicotinamide are identical by all spectroscopic means but exhibit equal molar rotations of opposite sign, as demonstrated by their ORD spectra (Figure 3). In contrast to the (*S*)-lactate dehydrogenase catalyzed reduction, the K_m value for pyr-nicotinamide corresponds well with that of pyruvate itself, whereas again the maximum velocity is rather low (0.07%, Table I).

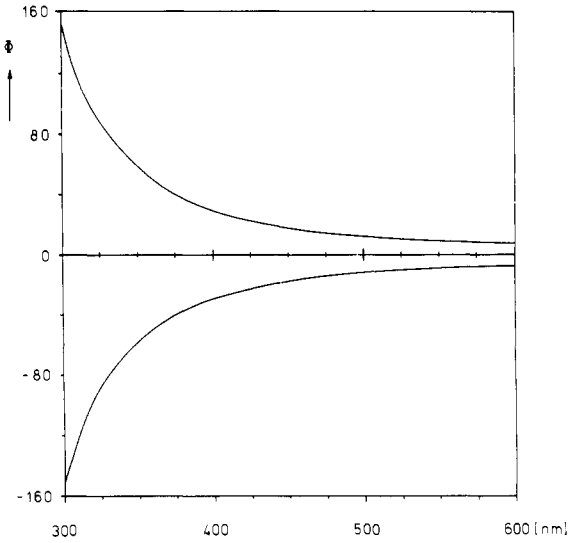

FIGURE 3: ORD spectra of (*S*)-lac-nicotinamide (upper curve) and (*R*)-lac-nicotinamide (lower curve), both 26.2 mM in water.

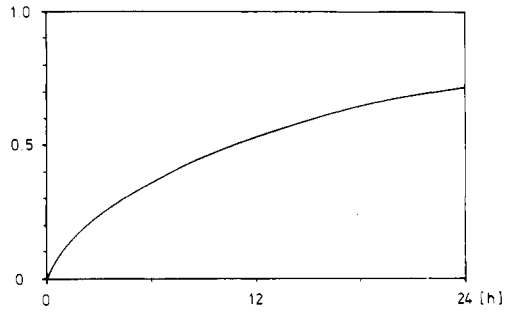

FIGURE 4: Time course of the conversion of 2.1 mM (*S*)-lac-NAD$^+$ in the presence of 20 μM pig heart lactate dehydrogenase in 1 M glycine buffer containing 0.4 M hydrazine (pH 9.5). Ordinate: relative increase of the 366-nm adsorption, 1.0 corresponding to 100% conversion to the hydrazone of pyr-NADH based on ϵ_{366} 3300 cm^2/mmol.

(*A*) *Investigations with (S)-Lactate Dehydrogenase.* Both (*S*)- and (*R*)-lac-NAD$^+$ are competitive inhibitors of pig heart lactate dehydrogenase with respect to NAD$^+$. However, the *S* diastereomer binds twice as tightly, K_i = 1.5 mM as compared to 3.0 mM. When the enzyme is added to a solution containing (*S*)-lac-NAD$^+$ alone in glycine–hydrazine buffer (pH 9.5), formation of the typical 340-nm absorption of dihydropyridines is observed. Figure 4 shows the time course of this reaction. The product formed could be identified as the hydrazone of pyr-NADH by ion-exchange chromatographic comparison with an authentic sample prepared from pyr-NADH itself. Within the detection level (about 2%) no other products could be found. Particularly, neither pyr-NAD$^+$ nor lac-NADH were present in the reaction mixture (Figure 5). The elution behavior of these compounds has previously been determined (Kapmeyer et al., 1976). The maximum velocity of this redox reaction only reaches 0.03% of the rate of the lactate oxidation by NAD$^+$. The Michaelis constant, however, shows an intermediate value between those of lactate and NAD$^+$ (Table I).

Addition of (*S*)-lactate causes a competitive inhibition of the redox reaction with an inhibition constant of 2 mM. Similarly, the rate is progressively decreased by increasing amounts of NAD$^+$ with a residual activity of 60% at equimolar amounts of the coenzyme and its analogue. With (*R*)-lac-NAD$^+$ in buffer alone, no reaction is observed upon addition of the enzyme.

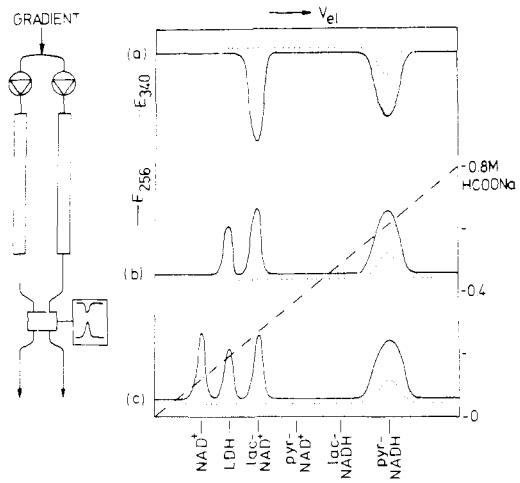

FIGURE 5: Identification of products from the intramolecular redox reaction in (*S*)-lac-NAD$^+$ catalyzed by (*S*)-lactate dehydrogenase. Simultaneous chromatography of the reaction mixture (b) and reference samples (a) in two parallel columns. (c) Reaction carried out in presence of NAD$^+$.

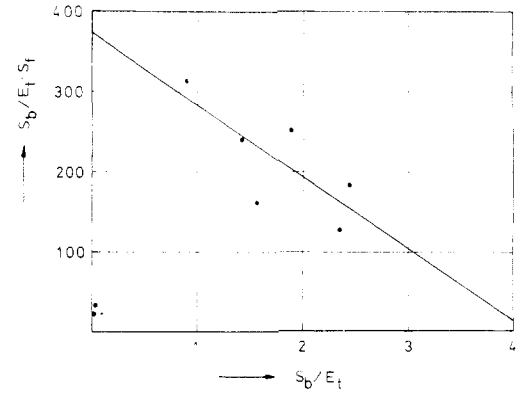

FIGURE 6: Scatchard plot of the binding of (*S*)-[3-^{14}C]lactate to pig heart lactate dehydrogenase in the absence of coenzyme, as studied by equilibrium dialysis.

Binding of (S)-Lactate in the Absence of Coenzyme. Binding of (*S*)-[3-^{14}C]lactate to pig heart lactate dehydrogenase in Tris buffer was studied in the absence of coenzyme by equilibrium dialysis. The enzyme had been treated with charcoal prior to these experiments in order to remove any tightly bound coenzyme (Wieland et al., 1962). Figure 6 shows a Scatchard plot (Scatchard, 1948) of the data which had to be corrected for the intrinsic volume of the protein due to the high enzyme concentration necessarily applied. The values at very low saturation have been disregarded for the calculation of the dissociation constant (Deranleau, 1969), 11 ± 2 mM based on 4.1 identical binding sites.

Coenzyme Analogous Reactions of 5-Methyl-NAD$^+$ and 5-Methyl-NADH. 5-Methyl-NAD$^+$ and 5-methyl-NADH were prepared with slight modifications, as originally described by Kaplan and co-workers (Walter and Kaplan, 1963). Both coenzyme analogues were found to substitute either NAD$^+$ or NADH in the presence of (*S*)-lactate or pyruvate, respectively. The kinetic constants are summarized in Table I. The maximum velocities of these reactions are less than 0.1% of the rate when the natural coenzymes are used. A contamination, however, with NAD$^+$ or NADH, which are coeluted from ion-exchange columns, accounting for these reactions can be excluded, because the chromatographed product was repeatedly digested with the highly specific *Neurospora* nucleosidase (Walter and Kaplan, 1963) and each time rechromatographed. Moreover, the hydride transfer was followed to more than 15% completion, at which point the rate was still unchanged.

(*B*) *Investigations with* (*R*)-*Lactate Dehydrogenase.* Both (*R*)- and (*S*)-lac-NAD$^+$ are competitive inhibitors of the *Lactobacillus* enzyme with respect to NAD$^+$, the *S* diastereomer being somewhat more potent, K_i = 1.8 mM as compared to 3.0 mM. In contrast to the *S*-specific enzyme, no reaction is observed after the addition of *Lactobacillus* or *Limulus* lactate dehydrogenase to either one of the analogues alone or in presence of (*R*)-lactate. A substrate analogous reaction after the addition of NAD$^+$ cannot fully be excluded. Its rate, however, is so low that a quantitative evaluation of the data is impossible.

Discussion

Pyr-nicotinamide has been shown to be active as a substrate analogue with (*S*)- and (*R*)-lactate-specific dehydrogenase yielding the two stereoisomers (*S*)- and (*R*)-lac-nicotinamide. The very low rate of these reductions (1/5000), in the case of the pig heart enzyme as compared to pyruvate, is in good agreement with values for other bulky pyruvic acid derivatives, as shown by Holbrook for α-nitrophenyl pyruvate (Holbrook and Stinson, 1973). The substituent is supposed to hinder a conformational change to an activated ternary complex. For an (*R*)-lactate-specific dehydrogenase, to our knowledge, pyr-nicotinamide is the first bulky substrate analogue that has been studied. The relative decrease in the rate (1/1440) is not quite as drastic as in the case of the *S*-specific enzyme, suggesting a less constrained environment in the substrate-binding domain. In (*S*)-lactate dehydrogenase, this constraint arises from a peptide loop that folds down over the active cleft in ternary complexes which may not be the case in the *R* enzyme (Holbrook et al., 1975). The complete conversion of pyr-nicotinamide to the lactate derivatives and its purification by ion-exchange chromatography (Trommer et al., 1976) exclude a contamination with pyruvate to account for these slow reactions.

Conversion of (*S*)- and (*R*)-lac-nicotinamide to the corresponding NAD$^+$ derivatives went exceedingly well. We have generally found the pig brain glycohydrolase to be superior to the often used calf spleen enzyme when 5-substituted nicotinamide derivatives are to be converted (Trommer et al., 1975; Kapmeyer-Nalik, 1973). Due to the acidic side chain in our analogues, separation from contaminating NAD$^+$ is easily achieved by ion-exchange chromatography.

The major result of this investigation is the finding that pig heart lactate dehydrogenase catalyzes a hydride transfer in (*S*)-lac-NAD$^+$ under formation of pyr-NADH (isolated as its hydrazone), whereas the (*R*)-lactate-specific enzymes from *Lactobacillus leichmannii* and *Limulus* do not catalyze this reaction in the *R* diastereomer. Addition of hydrazine as being done in the normal assay of the enzyme in the forward reaction (Walter and Kaplan, 1963) is necessary to shift the highly unfavorable equilibrium (Holbrook et al., 1975).

When pyr-NADH had been studied, the hydride transfer in the presence of (*S*)-lactate dehydrogenase was found to proceed *intra*molecularly, as revealed by competition experiments with pyruvate and NADH (Kapmeyer et al., 1976). In that reaction, addition of pyruvate had no effect on the rate. Lactate, however, is a competitive inhibitor in this case (K_i = 2 mM). In an *inter*molecular reaction in which one molecule of (*S*)-lac-NAD$^+$ acts as coenzyme and a second one with its side chain as substrate, (*S*)-lactate should successfully compete with the bulky "substrate" (*S*)-lac-NAD$^+$ and the rate should be increased. A likely explanation for the observed inhibition by lactate would be the binding of lactate in the absence of

coenzyme which has occasionally been reported in the literature but seems to have no significance in the reaction kinetics (Holbrook et al., 1975). Once lactate is bound, the substrate-binding domain is blocked and, consequently, binding of the combined coenzyme-substrate analogue (*S*)-lac-NAD$^+$ is inhibited. The dissociation constant of (*S*)-lac-NAD$^+$, as determined kinetically by using the analogue as an inhibitor of the normal NAD$^+$-dependent (*S*)-lactate oxidation ($K_d \approx K_i$ = 1.5 mM), would allow for the observed inhibition when lactate binding was of the same order of magnitude. Determination of the inhibition constant was possible because the conversion of (*S*)-lac-NAD$^+$ to pyr-NADH is so slow that it can be neglected. The dissociation constants for (*S*)-lactate, as quoted in the literature, vary from 1 to 100 mM (Takenaka and Schwert, 1956; Mosbach et al., 1972; Südi, 1970). The error in our estimation by equilibrium dialysis may be larger than the standard deviation (11 ± 2 mM) due to the necessary correction as pointed out under Results, but the value could explain the observed inhibition. The equilibrium dialyses were carried out in Tris and not in phosphate buffer because phosphate may, like sulfate (Adams et al., 1973), bind to arginine-171 in the apoenzyme and may, therefore, decrease the apparent lactate binding. A similar competitive inhibition by pyruvate of the hydride transfer in pyr-NADH could never be observed due to the extremely tight binding of the latter (K_d = 1.2 × 10^{-7} M) (Kapmeyer et al., 1976), whereas (*S*)-lac-NAD$^+$ exhibits a value comparable to NAD$^+$ itself and other oxidized coenzyme analogues. Addition of NAD$^+$, like (*S*)-lactate, led to a decrease in the rate of hydride transfer in (*S*)-lac-NAD$^+$ as expected for an *intra*molecular reaction. (*S*)-Lac-NAD$^+$ and NAD$^+$ are inhibitors of one another (see above) competing for the same binding site. Again, for steric reasons an *inter*molecular reaction should have been enhanced. Moreover, the theoretical products from such an *inter*molecular process, the hydrazone of pyr-NAD$^+$ and (*S*)-lac-NADH, have not been observed (Figure 5). Judging from a three-dimensional model of the enzyme, as determined by X-ray analysis (Eventoff et al., 1975, 1977), a reaction between two 5-substituted analogues may safely be excluded anyway. There is no space for two substrate molecules in their binding domain, which is in accordance with the (*S*)-lactate inhibition. (*S*)-Lac-NAD$^+$ itself, however, can nicely be fitted in the active pocket in a conformation allowing for the necessary interactions with histidine-195 and arginine-171 (Rossmann and Eventoff, private communication). Indeed, the enzyme and (*S*)-lac-NAD$^+$ have been cocrystallized, and the crystals exhibit the same morphology as other known ternary complexes (Eventoff et al., 1975). An X-ray analysis is presently being conducted at Purdue University. Even the methylene spacer does not seem to interfere significantly with groups of the enzyme. The results of Kaplan and co-workers that 5-methyl-NAD$^+$ and 5-methyl-NADH do not function as coenzymes in the presence of the substrates lactate or pyruvate (Walter and Kaplan, 1963), therefore, seemed rather puzzling. This investigation shows that these analogues, in fact, are active compounds with maximum rates, however, similar to those of our *intra*molecular reactions. In his experiments Kaplan had not used high enough enzyme concentrations to observe these reactions which would explain the discrepancy (Kaplan, private communication). What would be the explanation for the low rate of the *intra*molecular hydride transfer? The similar rates of the coenzyme analogous reaction with the 5-methyl derivatives would suggest that the orientation of the substrate part of (*S*)-lac-NAD$^+$ is just not optimal for hydride transfer. However, as pointed out before (Kapmeyer et al., 1976), the dissociation of the product complex in the natural reaction is

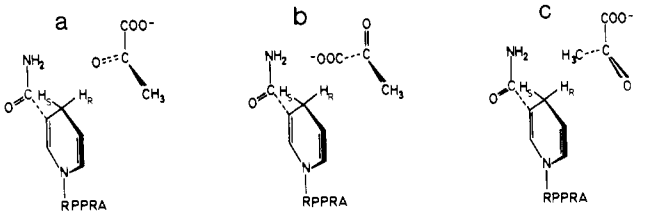

FIGURE 7: Possible arrangement of pyruvate in the active centers of (*S*)- and (*R*)-lactate-specific dehydrogenases allowing for the transfer of the *pro-R* hydrogen from the dihydropyridine ring in all cases: (a) (*S*)-lactate dehydrogenase according to X-ray data for ternary inhibitor complexes; (b) and (c) as postulated for (*R*)-lactate dehydrogenase.

of compulsory order. The apparent dissociation constant of the coenzyme in ternary complexes is much smaller than in the corresponding binary complex. In our case, the tightly binding pyr-NADH has to dissociate from a "ternary" complex which may be rate limiting. Moreover, the differences in rate between the natural reaction and our model system may actually be smaller. In preliminary experiments, Dr. Görisch of the University of Hohenheim has found a less than 500-fold decrease in rate in stopped-flow experiments during the first 500 ms of the progress curve.

As mentioned above, the (*R*)-lactate-specific dehydrogenases do not catalyze a hydride transfer in either one of the lac-NAD$^+$ diastereomers. Just as (*S*)-lactate dehydrogenase, these enzymes are specific for the so-called A side of the dihydropyridine ring under transfer of the *pro-R* hydrogen to pyruvate yielding (*R*)-lactate instead of the *S* stereoisomer (Dennis and Kaplan, 1960). The necessarily different arrangement of pyruvate in the active centers of the two types of enzymes may be brought about by either rotation of the C_2–C_3 bond with the carboxyl group and then pointing down as shown in Figure 7b or by a complete turn of the substrate by 180°. The first of the possible arrangements has been realized in (*R*)-lac-NAD$^+$. However, no hydride transfer occurs, leaving arrangement c in Figure 7 more likely. (*S*)-Lac-NAD$^+$ was found to be a somewhat stronger inhibitor of the (*R*)-lactate dehydrogenase than the *R* diastereomer. Assuming the latter arrangement to be correct, (*S*)-lac-NAD$^+$ could, indeed, show some interaction of the carboxyl group in the side chain with a possible active-center arginine, whereas the substrate moiety of (*R*)-lac-NAD$^+$ would not fit at all. The synthesis of a new coenzyme–substrate analogue for these enzymes in which the substrate is attached via a spacer to the carboxamide function of the nicotinamide ring is presently being carried out in our laboratory. If indeed in (*R*)-lactate-specific enzymes the substrate binding is reversed, we would then expect intramolecular hydride transfer to occur. However, depending on the orientation of the carboxamide function, analogues with various chain lengths will have to be investigated, as pointed out by Karabatsos et al. (1977) in the case of alcohol dehydrogenase.

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