Model Dehydrogenase Reactions. Catalysis of Dihydronicotinamide Reductions by Noncovalent Interactions†

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ABSTRACT: Carboxylate, pyrophosphate, and hydroxyl groups can accelerate the nonenzymatic rates of dihydronicotinamide reductions via intramolecular noncovalent interactions. The accelerations by the negatively charged carboxylate and pyrophosphate groups occur in nonpolar solvents but the effect of the hydroxyl groups occurs both in aqueous and nonaqueous solution. The largest effects are observed for neighboring carboxylate groups in nonpolar solvents. For example, the second-order rate constant for the reduction of *N*-methylacridinium ion by *N-cis*-2′-carboxycyclopentyldihydronicotinamide in acetonitrile is 1000 times more rapid than the rate constant for the corresponding methyl ester. Apparently, the negatively charged carboxylate stabilizes the partial positive charge which develops on the nicotinamide moiety in the transition state. The conclusion that the negatively charged pyrophosphate can enhance dihydronicotinamide reductions is based on the observation that reduced β-nicotinamide adenine dinucleotide (β-NADH) reduces *N*-methylacridinium ion 30-fold faster in methanol than in aqueous solution, while reduced α-nicotinamide adenine dinucleotide (α-NADH) reduces the oxidant only seven times faster in methanol than in water. The pyrophosphate group enhances the reaction rates of both anomers by a distance-dependent field effect. The magnitude is greater for the β anomer because the pyrophosphate and nicotinamide moieties are nearer neighbors in this anomer. The rate accelerations produced by hydroxyl groups of alcohols are not as great as those observed for carboxylate groups in nonpolar solvents. In aqueous solutions, α-NADH reduces three different oxidants ten times more rapidly than β-NADH. In acetonitrile, synthetic dihydronicotinamides containing hydroxyl groups increase the rate sixfold. These modest accelerations with the neutral hydroxyl groups emphasize the importance of a negatively charged group in order to achieve large enhancements in nonaqueous solutions.

Recent x-ray crystallographic studies on NAD^+[1]-dependent dehydrogenases are providing increasing information on the important noncovalent interactions utilized by these enzymes to catalyze direct and stereospecific hydrogen transfer between the coenzyme and the substrates (Adams et al., 1973; Holbrook et al., 1975; Eklund et al., 1974; Bränden et al., 1975; Dalziel, 1975). Nonenzymatic dihydronicotinamide reductions can play an important complementary role in probing dehydrogenase mechanisms by providing chemical precedents for catalytic interactions deduced from the x-ray structure as well as suggesting new interactions not immediately obvious from the structural information.

X-ray studies of dogfish lactate dehydrogenase (Holbrook et al., 1975) and horse liver alcohol dehydrogenase (Bränden et al., 1975), along with other experimental approaches (Woenckhaus et al., 1969; Sigman, 1967), have clearly indicated that acid–base catalysis and metal-ion catalysis, respectively, play important roles in activating the substrate for its reversible oxidation–reduction. Nonenzymatic reactions have provided unambiguous chemical precedents for these modes of catalysis (Shinkai and Bruice, 1973; Creighton and Sigman, 1971). The identification of the noncovalent interactions between the dehydrogenase and nicotinamide moiety has been less definitive. An early x-ray study had indicated that a glutamyl residue is in close proximity to the nicotinamide moiety of the coenzyme in an inactive ternary complex of dogfish lactate dehydrogenase (Adams et al., 1973). Although more recent sequence information has forced modification of this assignment (Taylor, 1977), that initial study motivated us to determine if neighboring carboxylate groups could accelerate nonenzymatic dihydronicotinamide reductions. Substantial catalysis by carboxylate groups might be anticipated in these reactions if the nicotinamide moiety develops net positive charge in the transition state. Electrostatic interaction with the neighboring negatively charged carboxylate groups should lower the net free energy of activation of the reaction.

To test this possibility, a series of *N*-benzyldihydronicotinamide derivatives, including *N*-2′-carboxybenzyldihydronicotinamide (Ib), was synthesized and their rates of reduction of *N*-methylacridinium (II) were examined (eq 1) (Hajdu and Sigman, 1975). It was observed that *N*-2′-carboxybenzyldihydronicotinamide (Ib) nonenzymatically reduces *N*-methylacridinium ion (II) in acetonitrile 150 times faster than *N*-benzyldihydronicotinamide (Ia), eq 1 (Hajdu and Sigman, 1975).

In the present communication, we wish to report that an even greater acceleratory effect of an intramolecular carboxylate group in a dihydronicotinamide reduction can be demonstrated with *N-cis*-2′-carboxycyclopentyldihydronicotinamide (V), a derivative where the carboxylate moiety is more rigidly fixed near the nicotinamide moiety than in Ib. We have also discovered that negatively charged pyrophosphate groups and the hydroxyl group of alcohols enhance dihydronicotinamide reductions by noncovalent interactions. The pyrophosphate group, like the carboxylate, probably enhances the reaction rate via electrostatic stabilization of the transition state. The

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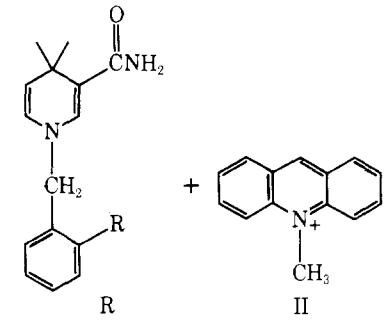
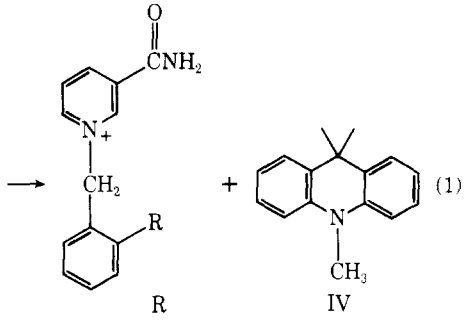
[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; NMNH, reduced nicotinamide mononucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NMR, nuclear magnetic resonance; IR, infrared; UV, ultraviolet.



R II

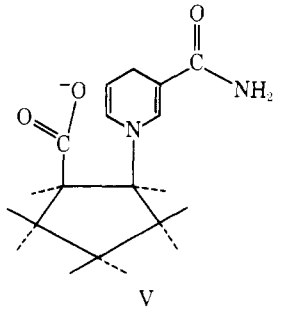
Ia H
Ib 2′-COO^-
Ic 2′-C(=O)OCH_3



R IV (1)

IIIa H
IIIb 2′-COO^-
IIIc 2′-C(=O)OCH_3

origin of the kinetic effect of the hydroxyl group is not clear but its magnitude is less significant than that of the carboxylate or pyrophosphate groups.



V

Experimental Procedures

Materials. The reduced α- and β-nicotinamide adenine dinucleotides were obtained commercially. α-NADH disodium salt (Sigma Chemical Co., "grade II"), which contains 15–20% β-NADH, was assayed spectrophotometrically prior to each kinetic run using horse liver alcohol dehydrogenase (Worthington) with freshly distilled acetaldehyde at pH 7.0 (Okamoto, 1971). Rate constants were corrected for the variable amounts of β-NADH present. β-NADH disodium salt (Calbiochem, "A grade") was used without further purification. α-NMNH was prepared by enzymatic cleavage of the dinucleotide (Suzuki et al., 1967). β-NMNH sodium salt (Sigma Chemical Co., type III) was used without further treatment.

N-Alkyldihydronicotinamides were prepared from the oxidized precursors by dithionite reduction using the procedure of Mauzerall and Westheimer (1955). The products were recrystallized from ethanol–water, and the structure of each compound was confirmed by NMR, IR, UV, and mass spectra. The purity of the compounds was established by thin-layer chromatography (silica gel–methanol and silica gel–chloroform) and paper chromatography (2-propanol–ammonia–water, 7:1:2; and 1 M ammonium acetate–ethanol, 3:7). Melting points are poor criteria of purity due to decomposition. The analytical data on the synthetic pyridinium salts and the reduced dihydronicotinamide analogues are summarized in Table I.

All oxidized nicotinamide precursors other than some of the *N*-benzylnicotinamide derivatives were prepared by the method of Lettré et al. (1953). The advantage of this synthetic procedure is that the nicotinamide ring in the product is present in the same stereochemical position as the amino group in the parent primary amine. The oxidized nicotinamides derived from *cis*- and *trans*-2-aminocyclopentanol (McCasland and Smith, 1950), the methyl ester of *cis*-2-aminocyclopentylamine (Aldrich) and *p*-methoxybenzylamine (Aldrich), and *exo*-3-amino-*exo*-2-norborneol (Simons, 1973) were prepared in this way.

The oxidized *N*-benzylnicotinamide derivatives were prepared by alkylation of nicotinamide with the appropriate benzyl halide in acetone. The benzyl halides used included benzyl chloride (Matheson, Coleman and Bell), α-bromo-*o*-xylene (Aldrich), *p*-cyanobenzyl bromide (Aldrich), and α-bromo-*p*-xylene (Matheson, Coleman and Bell), α-bromo-α′-hydroxy-*o*-xylene (obtained by borohydride reduction of α-bromo-*o*-toluic acid bromide, Brown et al., 1956).

The dihydronicotinamides containing free carboxylate groups were most conveniently prepared by hydrolysis of the corresponding methyl esters. The complete synthesis of the 2′-carboxybenzyldihydronicotinamide involved conversion of *o*-toluic acid to acid chloride with thionyl chloride, followed by formation of the methyl ester on addition of methanol. Photobromination in refluxing carbon tetrachloride (Eliel and Rivard, 1952) gave methyl α-bromo-*o*-toluate which was then reacted with excess nicotinamide in acetone. White needles of *N*-2′-carbomethoxybenzylnicotinamide bromide (mp 197 °C) separated after 15 days at room temperature (70% yield). Recrystallization from methanol–ether gave pure *N*-2′-carbomethoxybenzyldihydronicotinamide bromide. The molecular weight determined by Volhard titration for bromide (Kolthoff and Sandell, 1952) yielded a value of 351.60 g-equiv. The corresponding dihydronicotinamide (mol wt 272.31) was obtained by reduction with dithionite (λ_{max}^{MeOH} = 354 nm; mass spectrum P = 272).

The hydrolysis of *N*-2′-carbomethoxybenzyldihydronicotinamide to the corresponding carboxylate salt was accomplished by suspending 200 mg of the ester in 40 mL of 0.5 N aqueous barium hydroxide solution. Vigorous stirring for 3 h at room temperature resulted in an almost completely clear yellow solution containing the barium salt of the *N*-2′-carboxybenzyldihydronicotinamide. Solid ammonium carbonate (1.4 g) was added and after 30 min of further stirring the white precipitate of barium carbonate was centrifuged off. The supernatant was then lyophilized and the residue was redissolved in a minimum volume (2–3 mL) of 1.0 M aqueous ammonia. The solution was then passed through a Sephadex G-15 column (2.4 × 22 cm), packed in 1.0 M aqueous ammonia, and eluted with the same solvent. The peak fractions, absorbing at 358 nm, were combined and a stoichiometric amount of potassium (or sodium) hydroxide was added. The solution was lyophilized for 3 days and gave a yellow hygroscopic semicrystalline residue (λ_{max}^{MeOH} = 356 nm). Its NMR spectrum in 0.01 N NaOD/D_2O was as expected for the desired product. It gave a single fluorescent spot on paper chromatography (2-propanol–ammonia–water, 7:1:2) and on TLC (silica gel–methanol).

TABLE I: Analytical Data on the Oxidized and Reduced *N*-Alkylnicotinamide Derivatives.

Substitution on the nicotinamide ring	Oxidized nicotinamide halide	Mol wt by halide ion (Volhard) titration[a]	Mol wt calcd	Reduced dihydronicotinamide derivative	P^+ mass spectrum	Mol wt calcd
N-Benzyl	$C_{13}H_{13}N_2OCl$	248.90	248.71	$C_{13}H_{14}N_2O$	214	214.27
N-2′-Methylbenzyl	$C_{14}H_{15}N_2OBr$	307.55	307.20	$C_{14}H_{16}N_2O$	228	228.30
N-4′-Methylbenzyl	$C_{14}H_{15}N_2OBr$	307.48	307.20	$C_{14}H_{16}N_2O$	228	228.30
N-2′-Carbomethoxybenzyl	$C_{15}H_{15}N_2O_3Br$	351.60	351.21	$C_{15}H_{16}N_2O_3$	272	272.31
N-2′-Carboxybenzyl (hydrochloride)	$C_{14}H_{13}N_2O_3Cl$	293.55	292.72			
N-4′-Carbomethoxybenzyl	$C_{15}H_{15}N_2O_3Br$	351.00	351.21	$C_{15}H_{162}O_3$	272	272.31
N-4′-Carboxybenzyl (hydrobromide)	$C_{14}H_{13}N_2O_3Br$	338.00	337.18			
N-2′-Hydroxymethylbenzyl	$C_{14}H_{15}N_2O_2Br$	323.95	323.20	$C_{14}H_{16}N_2O_2$	244	244.30
N-4′-Cyanobenzyl	$C_{14}H_{12}N_3OBr$	318.00	318.18	$C_{14}H_{13}N_3O$	239	239.30
N-4′-Methoxybenzyl	$C_{14}H_{15}N_2O_2Cl$	279.24	278.75	$C_{14}H_{16}N_2O_2$	244	244.30
N-Cyclopentyl	$C_{11}H_{15}N_2OCl$	226.70	226.71	$C_{11}H_{16}N_2O$	192	192.26
N-cis-2′-Hydroxycyclopentyl	$C_{11}H_{15}N_2O_2Cl$	241.95	242.71	$C_{11}H_{16}N_2O_2$	208	208.26
N-trans-2′-Hydroxycyclopentyl	$C_{11}H_{15}N_2O_2Cl$	243.18	242.71	$C_{11}H_{16}N_2O_2$	208	208.26
N-cis-2′-Carbomethoxycyclopentyl	$C_{13}H_{17}N_2O_3Cl$	284.90	284.75	$C_{13}H_{18}N_2O_3$	250	250.30
N-exo-cis-2′-Hydroxy-3′-norbornyl	$C_{13}H_{17}N_2O_2Cl$	268.70	268.75	$C_{13}H_{18}N_2O_2$	234	234.30

[a] Kolthoff et al., 1952.

It could be stored at −20 °C as a solid for months. Before each kinetic experiment the compound was passed through a Sephadex LH-20 column (1.6 × 100 cm) in anhydrous methanol. *N*-4′-Carboxybenzyldihydronicotinamide was prepared in an entirely analogous way.

N-Methylacridinium was prepared according to Mooser et al. (1972). The chloride and the tetrafluoroborate salts were obtained using the anion-exchange resin Bio-Rad AG 1-X2 (50–100 mesh). Deazaflavin (10-methyl-5-deazaisoalloxazine) was synthesized by the method of O'Brien et al. (1970). Chloranil (Eastman) was recrystallized from acetone (mp > 290 °C). Dihydroquinone (J. T. Baker) was recrystallized from ethanol (mp 173–174 °C). Diamide (Calbiochem) was used without further purification.

Methanol (Matheson, Coleman and Bell, reagent grade) was distilled from *N*-methylacridinium iodide. This procedure is absolutely necessary to remove nucleophilic impurities present in the solvent (Ritchie et al., 1967), which react with *N*-methylacridinium ion by adding to its electrophilic 9 position. Acetonitrile (Matheson, Coleman and Bell, bp 80.5–82.5 °C) was distilled from *N*-methylacridinium iodide and kept on molecular sieves (Linde 4A, Union Carbide Co.) for at least 36 h before use.

Methods. All spectra were obtained either with a Cary 14 or Zeiss PMQII spectrophotometer. These instruments were also used for the kinetic studies. Reaction rates with half-lives less than a minute were measured on a Durrum D110 stopped-flow spectrophotometer. All kinetic measurements were performed at 25.0 ± 1 °C.

Kinetic measurements in all solvents were carried out under pseudo-first-order conditions with at least a tenfold excess of reductant. The maximum concentration of oxidant ever employed was 2×10^{-5} M. The disappearance of *N*-methylacridinium ion could be readily measured using its strong absorption at 358 nm (ϵ = 26 000) or its longer but weaker absorption at 420 nm (ϵ = 4800). The 420-nm absorption band was used to monitor reactions in which the dihydronicotinamide was present at very high concentrations, because the reducing agent absorbs intensely in the 340–360-nm range. Alternatively, the formation of the *N*-methylacridan product could be followed at 290 nm where most other reaction components possess minimal absorption.

All reaction mixtures exhibited strict first-order kinetics over two half-lives when the dihydronicotinamide was present in large excess. For all the kinetics reported here, the observed pseudo-first-order rate constants exhibited a linear dependence on the dihydronicotinamide concentration up to 6×10^{-3} M. The dihydronicotinamides were completely stable during the time interval required for the kinetic measurements. Free-radical quenching agents such as dihydroquinone and 4-*tert*-butylcatechol had no effect on the measured reaction rates. Identical results were obtained whether the reaction had been carried out in the presence or absence of oxygen. When synthetic *N*-alkyldihydronicotinamides were used, methanolic solutions of the reductant were diluted into the aqueous reaction media. The rates in aqueous solution were insensitive to methanol concentrations ranging from 1 to 10%.

To ensure that trace impurities in the synthetic dihydronicotinamide preparations would not affect the observed kinetics, especially in anhydrous solutions, the dihydronicotinamide preparations were further purified by passage through a Sephadex LH-20 column using methanol as eluent. The peak fractions were diluted for the kinetic runs. The concentration of the dihydronicotinamides in these fractions could be determined either spectrally or by spectrophotometric titration with *N*-methylacridinium ion.

The sodium salts of NADH and its derivatives were solubilized using 0.1 M 18-crown-6 ether (PCR Inc., Gainesville, Fla.) in methanol. In control experiments, the crown ether had no effect, at these concentrations, on the reaction rates with neutral dihydronicotinamides.

Product Analysis. All reaction products were identified using authentic samples of the corresponding compounds. Chromatographic (paper and TLC) and spectroscopic (UV, NMR, and mass spectra) techniques were utilized. For mass spectral analysis, the reaction was carried out on a larger scale

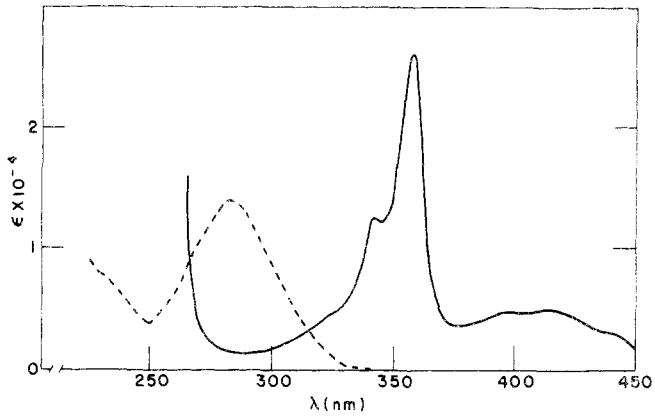


FIGURE 1: The absorption spectra of the oxidant (—) *N*-methylacridinium chloride and the reduction product (- - - -) *N*-methylacridan in anhydrous methanol.

and the products were isolated by extraction (Creighton et al., 1973) or chromatography on a Sephadex LH-20 column in methanol. By using methanol-d_4 as solvent, the products in the reaction mixture could be analyzed directly by NMR.

Results and Discussion

Suitability of N-Methylacridinium Ion as Oxidant. *N*-Methylacridinium ion was primarily used as the oxidant (Creighton et al., 1973) to evaluate effects of structural alteration on the reactivity of dihydronicotinamides for several reasons. They include (a) the ease of following the reaction due to the large spectral changes which accompany its stoichiometric conversion to *N*-methylacridan (Figure 1); (b) its rapid rate of reduction (e.g., Table VI) and hence the absence of complicating side reactions due to the decomposition of dihydronicotinamides (Creighton and Sigman, 1971; Johnston et al., 1963); (c) the direct transfer of the hydrogen from the dihydronicotinamide to the oxidant by a mechanism insensitive to free radical quenching agents; (d) the incorporation of the transferred hydrogen into a nonexchangeable position in the *N*-methylacridan product; (e) the stability of the products in the presence of oxygen; and finally (f) the solubility of *N*-methylacridinium ion in a wide variety of solvents.

Insensitivity of Dihydronicotinamide Reductions to Inductive Effects. Prior to assessing the importance of noncovalent interactions in accelerating dihydronicotinamide reductions, it was essential to demonstrate that the dihydronicotinamides could be effectively insulated from inductive effects. The data summarized in Table II show that the second-order rate constants for *N*-methylacridinium ion reduction are relatively insensitive to effects of substituents on the phenyl ring when a series of *N*-benzyldihydronicotinamides is used. Apparently, a methylene bridge sufficiently insulates the dihydronicotinamide moiety from electronic effects. Therefore, any kinetic effect exerted by a function which is insulated by at least a methylene group from the dihydronicotinamide moiety must be operating through space via a noncovalent interaction.

Acceleratory Effect of Carboxylate Groups. Two compounds were used to test the capacity of carboxylate groups to accelerate dihydronicotinamide reaction via noncovalent interactions. One compound was a derivative of *N*-benzyldihydronicotinamide, *N*-2′-carboxybenzyldihydronicotinamide (Ib). The other was a derivative of *N*-cyclopentyldihydronicotinamide, *N-cis*-2′-carboxycyclopentyldihydronicotinamide (V). In Table III, the impressive rate accelerations caused by

TABLE II: Second-Order Rate Constants for the Reduction of *N*-Methylacridinium Ion by a Series of Substituted *N*-Benzyldihydronicotinamides.

N-Benzyldihydronicotinamide substituent	$k_2^{25\,°C}$ (L mol^{-1} s^{-1}) 0.05 M Tris (pH 8.0)	MeOH	Acetonitrile
H	550	180	65
4′-CN	180		26
4′-$COOCH_3$	270		42
4′-CH_3O	530		92
2′-CH_3	540		70
2′-$COOCH_3$	460	220	70

TABLE III: Neighboring-Group Effects in Carboxyl-Substituted *N*-Alkyldihydronicotinamide Reduction of *N*-Methylacridinium Ion.

Dihydronicotinamide	$k^{25\,°C}$ (L mol^{-1} s^{-1}) 0.05 M Tris (pH 8.0)	MeOH	Acetonitrile
N-Benzyl (Ia)	550	180	65
N-2′-Carbomethoxybenzyl (Ic)	460	220	70
N-2′-Carboxybenzyl, Na^+ salt (Ib)	1450	4 000	10 000
N-2′-Carboxybenzyl, K^+ salt (Ib)			15 000
N-4′-Carboxybenzyl	610	NR[a]	NR[a]
N-Cyclopentyl	2300	350	150
N-cis-2′-Carbomethoxycyclopentyl	200		36
N-cis-2′-Carboxycyclopentyl, Na^+ salt (V)	1950	22 000	30 000

[a] NR, no reduction.

the carboxylates in nonaqueous solutions relative to appropriate reference compounds are recorded. Using the methyl ester as a reference, the cyclopentyl derivative provides a larger rate acceleration than the *N*-benzyl derivative. This is probably due to the more rigid constraint of the carboxylate group to the dihydronicotinamide moiety in the former compound. Of particular interest is the extreme sensitivity of the reduction by Ib to traces of water added to acetonitrile. Increasing the water concentration in acetonitrile to 10% causes a decrease in the second-order rate constant from 10 000 L mol^{-1} s^{-1} to 1300 L mol^{-1} s^{-1}. Apparently, when the carboxylate becomes hydrated, it is inhibited in its approach to the dihydronicotinamide moiety.

Reduction by *N*-4′-carboxybenzyldihydronicotinamide is not observed in acetonitrile, since the carboxylate group of this derivative, like that of acetate, *o*-, and *p*-toluate, rapidly adds to the electrophilic 9 position of *N*-methylacridinium ion to form a stable nonreducible adduct. Although the rate of adduct formation with *p*-toluate is one-half that observed for reduction by Ib, no adduct formation is seen with Ib or V, probably because the carboxylate groups in these compounds are sterically hindered and therefore substantially less effective nucleophiles.

Support for this close association of the carboxylate group to the dihydronicotinamide moiety is apparent from the absorption spectra of the various dihydronicotinamides in different solvents (Table IV). The absorption band associated with the π to π^* transition of the dihydronicotinamide moiety is blue shifted for neutral derivatives in going to less polar solvents. However, for the reactive dihydronicotinamide de-

TABLE IV: Effect of *N*-Alkyl Substituent of Dihydronicotinamide on the Absorption Maxima in Different Solvents.

N-Alkyl Substituent	λ (nm) H_2O	MeOH	MeCN
N-Benzyl (Ia)	358.0	353.0	347.5
N-2′-Methylbenzyl	358.0	353.5	348.0
N-2′-Carbomethoxybenzyl	358.0	354.0	348.0
N-2′-Carboxybenzyl (Ib)[a]	358.0	356.0	352.0
N-4′-Carboxybenzyl[a]	358.0	353.5	348.0
N-Propyl	360.0	355.0	350.0
N-Cyclopentyl	361.5	356.6	349.0
N-*cis*-2′-Carbomethoxycyclopentyl	356.5	351.5	346.5
N-*cis*-2′-Carboxycyclopentyl (V)[a]	361.5	356.5	353.0

[a] Sodium salt.

TABLE V: Rates of Reduction of Tetrachlorobenzoquinone (Chloranil) by a Series of *N*-Benzyldihydronicotinamides.

Dihydronicotinamide	$k^{25\,°C}$ (L mol^{-1} s^{-1}) MeOH	Acetonitrile
N-Benzyl (Ia)	1100	1 900
N-2′-Carboxybenzyl, K^+ salt (Ib)[a]	3000	22 500
N-4′-Carboxybenzyl, K^+ salt[a]	2800	3 500

[a] With 0.001 M 18-crown-6.

TABLE VI: Rates of Reduction of Nonenzymatic Oxidants by Anomeric-Reduced Pyridine Mono- and Dinucleotides[a]

Dinucleotide	Oxidant	$k_2^{25\,°C}$ (L mol^{-1} s^{-1} [b])
α-NADH	*N*-Methylacridinium[c]	1300
β-NADH	*N*-Methylacridinium[c]	101[d]
α-NMNH	*N*-Methylacridinium[c]	900
β-NMNH	*N*-Methylacridinium[c]	42
α-NADH	Diamide[f]	84
β-NADH	Diamide[f]	5.7
α-NADH	Deazaflavin[e]	0.5
β-NADH	Deazaflavin[e]	0.048

[a] In 0.1 M phosphate buffer at pH 8.0. [b] All kinetic runs were carried out under pseudo-first-order conditions with up to 2×10^{-5} M oxidant. The concentration of the reduced dinucleotides was varied up to 2×10^{-3}. Within this concentration range all the semilogarithmic plots were strictly linear. The absolute values of the second-order rate constants are accurate within ±10%. [c] *N*-Methylacridinium chloride iodide and tetrafluoroborate were used interchangeably, with no anion effect on the rates. [d] Taken from Creighton et al. (1973). [e] 10-Methyl 5-deazaisoalloxazine was dissolved in dimethylformamide and diluted into aqueous buffer (5% DMF final concentration) (Brüstlein and Bruice, 1972). [f] *N,N,N′,N′*-Tetramethylazoformamide was dissolved in methanol and diluted into aqueous buffer (less than 1% methanol in the reaction mixture) (Brown, 1971).

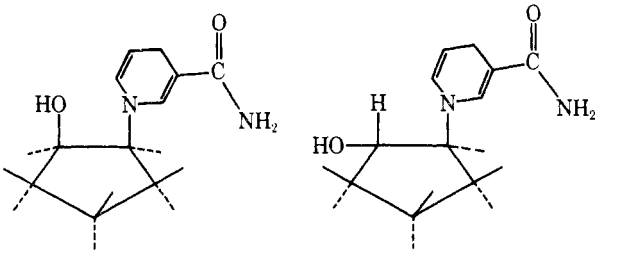
rivatives containing the carboxylate group, there is a pronounced red shift relative to neutral reference compounds in nonpolar solvents. The spectral perturbation is expected if a negative group is adjacent to the chromophore and partially opposes the blue shift attendant with transfer to a medium or lower polarity. Positively charged neighboring groups cause a blue shift (Shifrin, 1964).

The rate accelerations observed for dihydronicotinamide with neighboring groups are not restricted to the reduction of *N*-methylacridinium ion. The rate of reduction of chloranil is also enhanced when vicinal carboxyl groups are present (Table V). The effect is not as pronounced presumably due to less charge development on the nicotinamide moiety in the transition state with chloranil, a more potent oxidant than *N*-methylacridinium ion.

Acceleratory Effects of Hydroxyl Groups. α- and β-NADH, two epimeric forms of the coenzyme, differ in the stereochemistry at the anomeric carbon of the nicotinamide bearing ribose (Jacobson et al., 1973) and exhibit different rates of reduction of three different oxidants in aqueous solution (Table VII). The α anomer, in which the 2′-OH is cis to the nicotinamide, reduces these oxidants about ten times faster in aqueous solution than the naturally occurring β anomer, in which the 2′-OH is trans to the nicotinamide relative to the plane of the ribose ring. A comparable rate differential is observed with the α- and β-anomeric forms of reduced nicotinamide mononucleotide which are obtained by enzymatic hydrolysis of the corresponding coenzyme at the pyrophosphate linkage (Table VI). Therefore the major reason for the differential reactivity of the α- and β-NADH cannot involve interaction of the adenine and dihydronicotinamide. Since inductive effects in the two coenzyme forms are probably the same, the modest acceleratory effect is most likely the result of a noncovalent interaction present in one anomer and absent in the other.

Examination of molecular models of the α form reveals that the hydroxyl group on the 2′ carbon of the nicotinamide bearing ribose is in van der Waals contact with the nicotinamide. Space-filling models indicate that the nicotinamide cannot freely rotate in the α anomer. In the β-anomeric form, the hydroxyl group of the 2′ carbon of the ribose linked to the nicotinamide cannot achieve this same proximity relationship because it is trans to the nicotinamide relative to the plane of the ribose ring. The tentative conclusion which can be drawn from this series of observations is that the hydroxyl group of alcohols is capable of accelerating dihydronicotinamide reductions by noncovalent interactions.

We sought to test this tentative conclusion by synthesizing simpler dihydronicotinamides in which the stereochemical relationship in the two anomers would be maintained. We chose to synthesize the carbocyclic analogues of the corresponding furanoside derivatives (VI and VII) to eliminate a

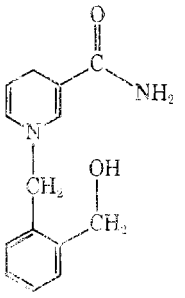


VI VII

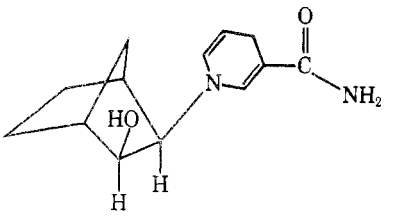
potential for stereolability. Two other derivatives which were synthesized and possessed comparable stereochemistry to the α-NADH derivative were *N*-2′-hydroxymethylbenzyldihydronicotinamide (VIII) and the bicyclic derivative (IX). These compounds show modest acceleratory effects when they are compared to the appropriate reference compounds (Table VII). In anhydrous solvents, the neighboring hydroxyl groups accelerate the dihydronicotinamide reductions by a factor of about six relative to a reference compound. These apparent rate

TABLE VII: Rates of Reduction of *N*-Methylacridinium Ion by a Series of *N*-β-Hydroxyalkyldihydronicotinamides.

Dihydronicotinamide	$k^{25\,°C}$ (L mol^{-1} s^{-1}) 0.05 M Tris (pH 8.0)	MeOH	Acetonitrile
N-cis-2′-Hydroxycyclopentyl (VI)	1000	770	650
N-trans-2′-Hydroxycyclopentyl (VII)	410	230	100
N-Cyclopentyl	2300	350	150
N-exo-cis-2′-Hydroxy-3′-norbornyl (IX)	2000		1500
N-2-Hydroxymethylbenzyl (VIII)	750		320
N-2′-Methylbenzyl	540		70



VIII



IX

enhancements result from a diminished solvent effect for compounds bearing hydroxyl groups. For the carboxylate derivatives, there was an absolute increase in the rate upon transfer from aqueous solution to acetonitrile.

These results indicate that a hydroxyl group can accelerate the rates of dihydronicotinamide reductions in nonpolar solvents presumably by facilitating the solvation of the positive charge on the dihydronicotinamide. They, however, underscore the more impressive effect that can be obtained with the negatively charged carboxylate and do not fully explain the source of the rate accelerations observed for α-NADH in aqueous solution. The pH independence of the rate of α-NADH reduction between 7 and 8 suggests that the 2′-OH is un-ionized. The identity of the rates in D_2O and H_2O tends to argue against specific solvent effects.

Acceleratory Effects of Pyrophosphate Groups. α- and β-NADH exhibit entirely different solvent effects than the other dihydronicotinamide derivatives with hydroxyl groups in the reduction of N-methylacridinium ion. When the disodium salt of either anomeric form was dissolved in methanol containing 0.1 M 18-crown-6, an absolute increase in the rate of reduction was observed relative to water. The rate constant for α-NADH increased sixfold in methanol relative to water, while that for the β anomer increased 30-fold. The absolute increase in the rate of reduction for both derivatives is probably due to stabilization of the incipient positive charge on the dihydronicotinamide by the negatively charged pyrophosphate. The larger enhancement for the β-NADH is due to the closer proximity of the negatively charged pyrophosphate and the nicotinamide. The pyrophosphate and nicotinamide are cis to one another relative to the plane of the ribose in the β anomer and hence nearer neighbors than in α-NADH.

These studies demonstrate the sensitivity of dihydronicotinamide reductions to microenvironment. Comparable studies will be undertaken using the carboxamide moiety as a neighboring group, since an asparagine residue appears to be conserved in five lactate dehydrogenases near the nicotinamide binding site (Taylor, 1977). Hopefully, the phenomena reported here will lead to a more detailed understanding of the mechanism of hydrogen transfer in other dehydrogenases.

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